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ACETYLCHOLINESTERASE INHIBITION AND INFORMATION PROCESSING IN THE AUDITORY CORTEX

ANNUAL SUMMARY REPORT

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Micropressure application of cholinergic agents to auditory cortex of the cat revealed selective muscarinic modification of background and tone-evoked discharges of single neurons. A real-time algorithm for recording from several single neuron simultaneously was developed, as was a behavioral model exhibiting rapid discriminative learning using rewarding brain stimulation.

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SUMMARY

As a foundation for determining the effects of cholinesterase inhibition on information processing and physiological plasticity in auditory cortex, the effects of cholinergic agonists and antagonists upon neuronal activity in the auditory cortex of the cat were studied by micropressure application of acetylcholine, beta-methacholine, atropine, and scopolamine. Single unit or unit cluster discharges were recorded while pure tone stimuli were presented in the presence or absence of cholinergic agents. Most cells were affected by one or more agents. Both background and evoked activity for the same cell could be altered in different ways. Furthermore, different components of the pattern of neuronal responses to acoustic stimuli were differentially sensitive to cholinergic substances. The effects appear to be mediated by muscarinic mechanisms. Exhaustive quantitative analysis of neuronal data is considered essential in order to understand cholinergic actions in the cortex. Computer-based real time waveform sorting programs have been devised to permit simultaneous recording of several single unit discharges in order to greatly facilitate data acquisition and enable the investigation of cholinergic mechanisms in the cortical physiological plasticity which develops during behavioral learning. A behavioral model was created in which rapid discriminative classical conditioning of the pupillary dilation response develops with the use of rewarding hypothalamic stimulation as the unconditioned stimulus. These findings provide the basis for investigation of acetylcholinesterase action in the auditory cortex.

FOREWORD

In the conduct of the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institue of Laboratory Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. PROBLEM

Anticholinesterase agents constitute a serious problem for chemical defense (1). Among their effects are direct disruption of normal brain function, with subsequent severe impairment of psychological and behavioral functions. Because these agents interfere with cholinergic mechanisms, which are a major component of the processing and transmission of information within the nervous system, their understanding requires rigorous, analytical characterization of cholinergic neuronal processes. Because adaptive behavior requires physiological plasticity, particularly in the cerebral neocortex, the effects of anticholinesterase agents on the nervous system must include the assessment of their effects on neocortical physiological plasticity as well as on information processing which precedes such plasticity. Unfortunately, virtually nothing is known about this problem. This project comprises the first systematic investigation of the role of cholinergic mechanisms in the processing of information and physiological plasticity in sensory cortex.

II. BACKGROUND

Acetylcholine (ACh) is widespread throughout the nervous system (2-8), and cholinergic transmission appears to be involved in many functions (2,9-12). A full understanding of cholinergic mechanisms has been of prime importance since the development and deployment of anticholinesterases as chemical warfare agents. This requires development and effective use of appropriate animal models. Recent development of a specific marker for cholinergic neurons, choline acetyltransferase (Chat) (4,13), has revealed that the cerebral cortex is widely innervated by cholinergic neurons. The major source of input is the nucleus cuneiformis of the reticular formation (4,14-20). Thus, an anatomical basis has been found for previous physiological reports that many neurons within the cerebral cortex are cholinoceptive (6).

Cholinergic innervation of the auditory cortex (16,20) is of particular interest because cholinergic agents interfere with auditory perception (2,21-23). Some effects on the processing of auditory information may involve the cholinergic olivocochlear bundle (24), which is involved in auditory attention (25-27), but this mechanism probably is not completely responsible for the known disruptive effects of cholinergic agents on such complex cognitive processes as breadth of attention for acoustic material (21,23), recognition of patterned acoustic sequences (28), learning and memory (9,24,29,30), or for causing auditory hallucinations (2,23,31,32). Thus, compounds which alter cholinergic transmission, in particular anticholinesterases, may affect the highest levels of the auditory system (7,23,34-37). This is supported by data which have established a second ascending auditory path which is cholinoceptive and is involved in the processing of stimulus significance or meaning.

Recent anatomical and physiological studies have established parallel ascending pathways within the auditory system of all mammals, including man. The better known path is the lemniscal line, which includes the tonotopically organized nuclei along the neuraxis: the ventral cochlear nuclei, the superior olivary complex, the central nucleus of the inferior colliculus, and the ventral nucleus of the medial geniculate body (8). This is paralleled by a nonlemnis-

cal path (39,40), in which the nuclei are not organized in a tonotopic fashion, and in which neurons have more labile response characteristics than do those of the lemniscal line (41). This nonlemniscal line includes the external nucleus of the inferior colliculus, the nucleus of the brachium of the inferior colliculus (peripeduncular nucleus), the magnocellular nucleus of the medial geniculate, and the medial posterior suprageniculate complex (40,42). The nonlemniscal pathway is cholinoceptive (35,40); the lemniscal path above the level of the brainstem is not cholinergic. However, virtually nothing is known regarding the function of cholinergic mechanisms in the nonlemniscal line or for that matter in the auditory cortex.

Cholinergic mechanisms could be involved in one or more of the following operations in the processing of information within the auditory cortex.

1. Relay of ascending information. This is unlikely because the lemniscal thalamic relay to the auditory cortex, the ventral medial geniculate nucleus, is not cholinergic.

2. Output of processed information to subcortical targets. This is unlikely because the output cells of the auditory cortex are not themselves cholinergic.

3. Intracortical processing of ascending information. This is consistent with findings that ACh has differential actions within the cortex, purportedly inhibiting neurons in upper layers and exciting cells in lower layers (36,37). The latter cells in layers V and VI are output neurons, and these are known to be cholinoceptive (4,5,35).

4. Regulation of cortical information processing. This role is consistent with several findings. First, cortical release of ACh depends on the state of arousal (9,10,36,43,44), which is a major variable in information-processing performance (24,45,46). Second, cholinergic projections are diffuse and widespread (3,4,15,35,), suggestive of a global regulatory function. Third, the nonlemniscal thalamocortical auditory system, centered on the magnocellular medial geniculate nucleus (MGm), is highly plastic (47-50), and is cholinoceptive (23). This system probably affects response plasticity in the auditory cortex, particularly as the thalamic auditory lemniscal relay, the ventral medial geniculate nucleus (MGv), is not plastic (47-55). Fourth, ACh in the brain promotes long lasting changes in neuronal excitability (5,6,56-60), a characteristic well-suited for the modulation of information processing.

These findings suggest that cholinergic mechanisms are likely to be involved in the regulation of information processing in the upper auditory system. Thus, attending to and understanding verbal messages in humans, irrespective of the particular voice which speaks them, may well involve cholinergic mechanisms in the auditory cortex. In fact, agents which interfere with cholinergic transmission also interfere with acoustic attention (21,22) and the recognition of patterned acoustic stimulus sequences (28) and can produce auditory hallucinations in man (2,23,31) (see also 61). Inhibition of acetylcholinesterase (AChE) would be expected to disrupt such processes.

There are other compelling reasons to study cholinergic mechanisms in the processing of acoustic information. Previous studies have been concerned mainly with the effects of cholinergic agents on brain systems which do not have clearly understood or defined functions. For example, the septohippocampal

projection appears to be cholinergic, but its functional role is unknown (62). Such approaches need to be supplemented with studies of the role of cholinergic mechanisms in systems whose functions are known. The auditory system has a clearly defined role. We propose to elucidate the role of cholinergic mechanisms on information processing in the auditory cortex, and in so doing provide a basis for an understanding of the mechanisms of action of deployable anticholinesterase compounds, including development of effective medical chemical defense measures. The auditory cortical system provides an excellent model for assessing potential chemical defenses because its normal informational input can be controlled by the experimenter, and this input can also be assigned desired significance by experimenter control of learning contingencies.

III. OBJECTIVES AND APPROACH

The technical objectives of this project are to determine the effects of anticholinesterase agents on information processing in the auditory cortex and to attempt to block or reverse these effects by pharmacological means. Because there have been no previous systematic studies of this problem, it will be necessary to first investigate cholinergic mechanisms in cortical information processing to provide a basis for the study of anticholinesterases. Measures of cortical information processing will be obtained from the discharges of single neurons to single tones and to sets of isointensity pure tones across the acoustic spectrum to yield frequency tuning functions. In addition, we will determine the effects of repeatedly applied cholinergic agents on the processing of acoustic stimulation whose significance is under experimental control, by assessing their actions on the development of discharge plasticity of frequency functions during the acquisition of a behavioral conditioned response.

This approach transcends standard cellular neuropharmacology approaches in at least three respects. Because of their singular nature, it is important that they be explicated at the outset. They concern 1) neurophysiology, 2) computer sorting of waveforms, and 3) behavioral learning.

A. Neurophysiology

We are characterizing the neurophysiological effects of cholinergic agents using techniques based upon sensory physiology and brain communication theory. This differs from common approaches which confine themselves to analysis of the mean rates of cellular discharge. It must be kept in mind that the pattern of discharge may be statistically independent of the rate of cellular firing. For example, pattern may change while overall rate is constant. Failure to analyze pattern may result in a lack of understanding of drug action on neurons. Although this method is time consuming, we strongly believe that it is essential to the aims of this project. Other less comprehensive methods are unlikely to lead to an understanding of the effects of agents on information processing.

Progress on this aspect of the project is provided in section IV.A.

B. Computer Waveform Sorting

Standard approaches record either from an unknown number of neurons, whose discharges are mixed together by the use of a low impedance recording electrode, or from one neuron at a time. The first so-called "multiple unit" technique has the advantage of providing for continual recording for an extended period of time and requires little expertise. However, it is disadvantageous because it cannot reveal different directions of uni-cellular firing (increasing or decreasing), and a single neuron can bias the entire recording and analyses. Furthermore, pharmacological effects may not be readily interpreted because of selective, opposing actions on different cells, an effect to which multiple unit recording is insensitive. An exception is when it can be demonstrated that a small "cluster" of discharges represents a physiologically and pharmacologically homogeneous population of neurons; we have been able to record from such clusters on occasion, as an interim solution to obtaining simultaneous recordings from single neurons.

On the other hand, the technique of recording from one cell at a time is critical in that it yields interpretable data. However, it is very time consuming and therefore costly. These limitations are particularly severe in experiments that require continuous recording from one cell for hours rather than minutes. In the case of this project, to which physiological plasticity is a central focus, data would be obtained from only one or two cells per behavioral training session.

Our solution to this problem is to develop a computer-based system for the recovery of single unit data from multiple unit records. By lowering the impedance of a recording micropipette, it is possible to obtain the discharges of several cells simultaneously. The alternative of using several separate recording electrodes is not desirable because of the low probability of recording single neurons from several electrodes over a long period of time, and because this would require several pipettes for application of pharmacological agents. Although a waveform-sorting computer had been advertised at the time the project was originally formulated, it was no longer a feasible choice at the start of this first year of research. Accordingly, we have been developing an online, real time system composed of several manufactured computers. This has necessitated de novo development of appropriate software, as well as the solution to many novel technical problems.

Progress on the development of computer-based waveform sorting is reviewed in section IV.B.

C. Behavioral Learning

In order to understand how anticholinesterases affect or impair the processing of meaningful information in auditory cortex, or elsewhere in the brain, the importance or significance of stimuli must be under control of the experimenter. This is possible via the use of behavioral training techniques, such as classical conditioning. However, it is insufficient to merely use a standard training situation, such as the eye blink or limb flexion. The demands of this and similar projects require that learning be sufficiently rapid to permit continuous recording from single neurons and that there be no adverse effects on

the animals. We have previously shown that the pupillary dilation conditioned response develops rapidly in the cat. We have now initiated use of positive, pleasurable electrical stimulation of the hypothalamus as a reward during training in undrugged subjects.

Progress on the development of a suitable behavioral learning situation is presented in section IV.C.

IV. RESULTS AND DISCUSSION

A. Neurophysiology

1. Introduction

Since virtually nothing is known about the effects of locally applied cholinergic agents on the background and evoked discharges of neurons in the auditory cortex, studies of the effects of anticholinesterases must be preceded by foundational studies of cholinergic involvement. The basic strategy is to characterize the effects of ACh, investigate the effects of antagonists, and determine whether muscarinic, nicotinic, or both types of systems are involved. During this first year of the project, following a period of obtaining and setting up essential equipment, these basic characterizations were largely accomplished.

2. Methods

a. Subjects and Surgical Preparation:

The subjects were healthy adult male cats, (3.1 - 5.2kg), maintained in a modern animal facility, under strict supervision of the University veterinarian. Animals were housed individually in approved stainless steel cages, with water and Purina cat chow available on an *ad libitum* basis. The animal facility provided a separate temperature-controlled room for the cats, which was placed on a 12-hour light-dark cycle (6:00 A.M.-6:00 P.M.). Animals were prepared for recording as previously reported in detail (e.g., 63). Briefly, general anesthesia was induced by pentobarbital sodium; a patent airway was ensured by intubation of the trachea with a pediatric endotracheal tube coated with a local anesthetic, under laryngoscopic control; body temperature was maintained with a thermostatically controlled hot water pad; and the animal was placed in a stereotaxic frame. Following a midline incision and clearing of the calvarium, a pedestal of dental acrylic was affixed to the skull; two metal spacer bolts were embedded within the pedestal to provide for atraumatic fixation of the head during recording sessions. Access to primary auditory cortex was provided by small burr holes. Following recovery in an incubator and antibiotic prophylactic therapy, the subject was allowed at least 1 week before recording began. Animals became accustomed to handling and were groomed by the experimenters.

On the day of a recording session, animals received gallamine triethiodide and were artificially ventilated; expired carbon dioxide levels were monitored with a Beckman (Fullerton, CA.) gas analyzer. This procedure was used in order to provide for foundational data to be obtained under conditions of strict acoustic control and to provide a basic characterization of cholinergic mechanisms

during the period while the learning preparation was under development (see sections III.C and IV.C.). The corneas were coated with ophthalmic ointment to preclude possible discomfort from drying, the subject was positioned on cushions; and the pedestal was bolted to be rigid support. Throughout the recording session, pupils were generally constricted and dilations could easily be elicited by incidental noise. The EEG was generally spindling or slow wave, indicative of quiet waking, drowsiness, or sleep. At the end of a session, the animal was returned to its home cage without problem and allowed 1-2 weeks between further recording sessions.

b. Recording, Experimental Protocol, and Data Analysis:

Multibarrel pipettes were prepared with NaCl for recording (impedances 5-10 megohms), and for the application of the agonists ACh (2 M in 0.2 M NaCl) or beta-methacholine (MCh, 10-20 mM) and for application of the antagonists, atropine (0.2-0.4 M) or scopolamine (15 mM). In general concentrations were consistent with those reported from previous published reports of the effects of cholinergic agents on sensory neocortex (6,34,37,58,59). A higher concentration of ACh than MCh was used because ACh is rapidly degraded by acetylcholinesterase (AChE) and larger initial concentrations are needed to have sufficient concentration at the receptors. MCh is resistant to degradation by AChE; this agent has easier access to muscarinic receptors and also remains in contact with receptors for longer periods than does ACh. Atropine and scopolamine concentrations were selected from the literature to produce effects. No attempt was made in these studies to determine the relative efficacy of atropine and scopolamine. We did not attempt dose response curves or determination of affinity (pA_2) for these antagonists in this work. Our intent was to determine the muscarinic nature of the response. Concentration effects maybe misleading as: 1) location of pipette to receptors varies, and 2) the effect is related to diffusion of the rates of drugs to the receptors. The maximum volume ejected was 1 nl, as determineed by examining the effect of maximum pressure ejection on randomly-selected micropipettes, using a compound microscope.

The acoustic delivery system was calibrated via a previously calibrated earpiece and speaker (AIWA) via a Hewlett-Packard (Palo Alto, CA.) wave analyzer under computer control. Stimuli were delivered to the contralateral ear. Frequencies were calibrated in 20 Hz steps from 0.05 kHz to 5.2 kHz, and in 200 Hz steps thereafter to 30.2 kHz. This system provided for presentation of pure tone frequencies of any desired intensity. Tone pulses were shaped with a rise/fall gate at 5 ms to preclude switching transients. Following calibration, the pipette assembly was inserted through a small dural slit with the aid of a surgical microscope. Electrical activity was recorded via a Dagan (Minneapolis, MN.) 2400 AC amplifier (band-pass 0.3-3.0 kHz). Unit activity was displayed on a storage oscilloscope, recorded on a Hewlett-Packard four channel instrumentation recorder, and led to a PDP 11/03 computer. The use of a raster display was helpful in determining best frequency, but is insufficiently precise; an online preanalysis system is under development to solve this problem. The rate of discharge usually was monitored by a rate meter and ink write out on a Grass (Quincy, MA.) model 7 polygraph. Cholinergic agents were administered by micropressure injection (Med Systems, Great Neck, NY.).

Following isolation of a single, stable waveform, indicative of recording from one neuron, the standard protocol was in general to determine the frequency

response of the cell to tonal stimulation (e.g., 300 ms, 75 db, 0.2-32.0 kHz). A frequency to which the cell responded was selected for study with drugs. This stimulus was repeatedly presented (N=20) during control periods and during drug administration. Repeated presentation of a tone is called a stimlulus "set." Following baseline recording, ACh or MCh was administered in ascending doses, intermixed with control periods, for durations of 30-60 s while background and evoked activity were recorded. The effects of a blocker (atropine or scopolamine) were then assessed. Following additional basic tests, the agonist and blocker were given simultaneously. In some cases in which continual isolation of a single cell was not possible, records were obtained from a neuronal "cluster", consisting of 2-3 waveforms. While such recordings were helpful and the results appeared to be similar to single unit findings quantitative analyses were restricted to single neurons.

Data from approximately 135 single cells have been obtained and subjected to statistical analysis at this time. The average rate and standard deviation of activity preceding each tone (e.g., 500 ms) and during each tone were computed for every condition. Furthermore, poststimulus time histograms (PSTH) were computed and inspected for various features, such as major periods of discharge ("peaks") and suppression ("valleys"). Each major feature was analyzed separately. Comprehensive evaluation of the data from approximately 60 neurons has been completed at this time. The terms "spontaneous", "background" and "prestimulus' activity are used interchangeably reflecting common use in sensory neurophysiology.

3. Results

A large proportion of neurons in the auditory cortex are affected by cholinergic agents. The effects differ according to whether the neuronal discharges ar background activity (sometimes called "spontaneous") or are evoked by tone. Furthermore, agonists and antagonists also yield different proportions of effects (Table 1). The data indicate that there is a substantial probability of obtaining a cholinergic effect on auditory cortical cells.

Table 1. Effects of Cholinergic Agents on Background and Evoked Discharges

	Type of Discharge	
Agent	Background	Evoked
Agonists	No. of Cells	No. of Cells
Change	39	38
Increase	21	14
Decrease	18	24
No Change	19	20
Total	58	58
Antagonist		
Change	27	23
Increase	8	5
Decrease	19	18
No Change	27	31
Total	54	54

Before considering in detail the effects of cholinergic agents, it is important to realize that the responses of neurons to sensory stimulation are not simply an increase or decrease in the rate of activity; rather, the pattern of response is also an important feature of information processing in the brain. The pattern of response is conveniently provided by post stimulus time histograms (PSTH). In addition to providing data on the pattern of response, the PSTH also provides data on the degree to which the cell is "driven" or "suppressed" by a stimulus. Figure 1 presents PSTH for nine neurons to 200 msec tones. The most common feature is a prominent discharge at the beginning of a tone ("on" response), as seen in B,C,D,E,H, and I. Often, there are "midtone" (also called "through") responses following the onset (e.g., B, E,H, and I). "Off" responses are also common features (F,G,H, and I); sometimes the tone produces a clear suppression of activity followed by a prominent off response (F and G). "On" responses had a latency following tone onset of 10-50 msec. "Midtone" responses are defined as discharges with latencies of 51-200 msec. "Off" responses are responses that occurred within 10-200 msec following termination of the tone. For cells which are not driven to such high degrees, a common response is a clear response during the tone followed by a gradual decrease to pretone (background) rates of discharge (A). The importance of tone pattern, as well as the difference between background and evoked activity, will be evident in the following discussion, in which cholinergic agents have differential effects on these aspects of neuronal discharges.

Cellular sensitivity to different doses is common. Figure 2 shows an increasingly depressive effect of MCh on the "off" response of a neuron in auditory cortex. The graph shows differences between the effects of three doses and the immediately preceding control period. PSTH are given above the graph. Note that MCh depresses the "off" response and also decreases to a lesser extent background activity. However, there is no change in the tone-evoked suppression during the tone itself. This suggests a differential effect of cholinergic agents on various aspects of the response to tone. More conclusive evidence is presented later.

Opposite effects of cholinergic agonists and antagonists were obtained. Figure 3 shows the PSTH of a cell in which tone elicited no activity except for a small "off" response. ACh itself appears to have no effect (but see quantitative analysis below). Atropine increased background firing and enabled the cell to respond during the tone; it also increased the "off" response. The importance of quantitative analyses of all aspects of the cell's activity, rather than confining analysis to mean rate and to visual inspection of PSTH, is evident from a summary of statistical analyses. In Figure 4 and subsequent bar graphs, the abscissa shows epochs of tone presentation (numbers represent control periods without drug). Figure 4 shows that ACh had no effect on either background or evoked activity for the response to tone; atropine clearly increased background and the response to tone. However, Figure 5, which summarizes data for the "off" response, reveals that ACh actually depressed the "off" response, and that the depression was greater for a higher dose of this agent (compare ACh 6.7 and 15.0 psi). The opposite effect of atropine for the "off" response is clearly evident ("Atropine 20.0") compared to the preceding and following control periods). Incidentally, note that the ACh depression was restricted to evoked activity for the off response; ACh had no effect on the discharges during the tone and had no effect on background activity.

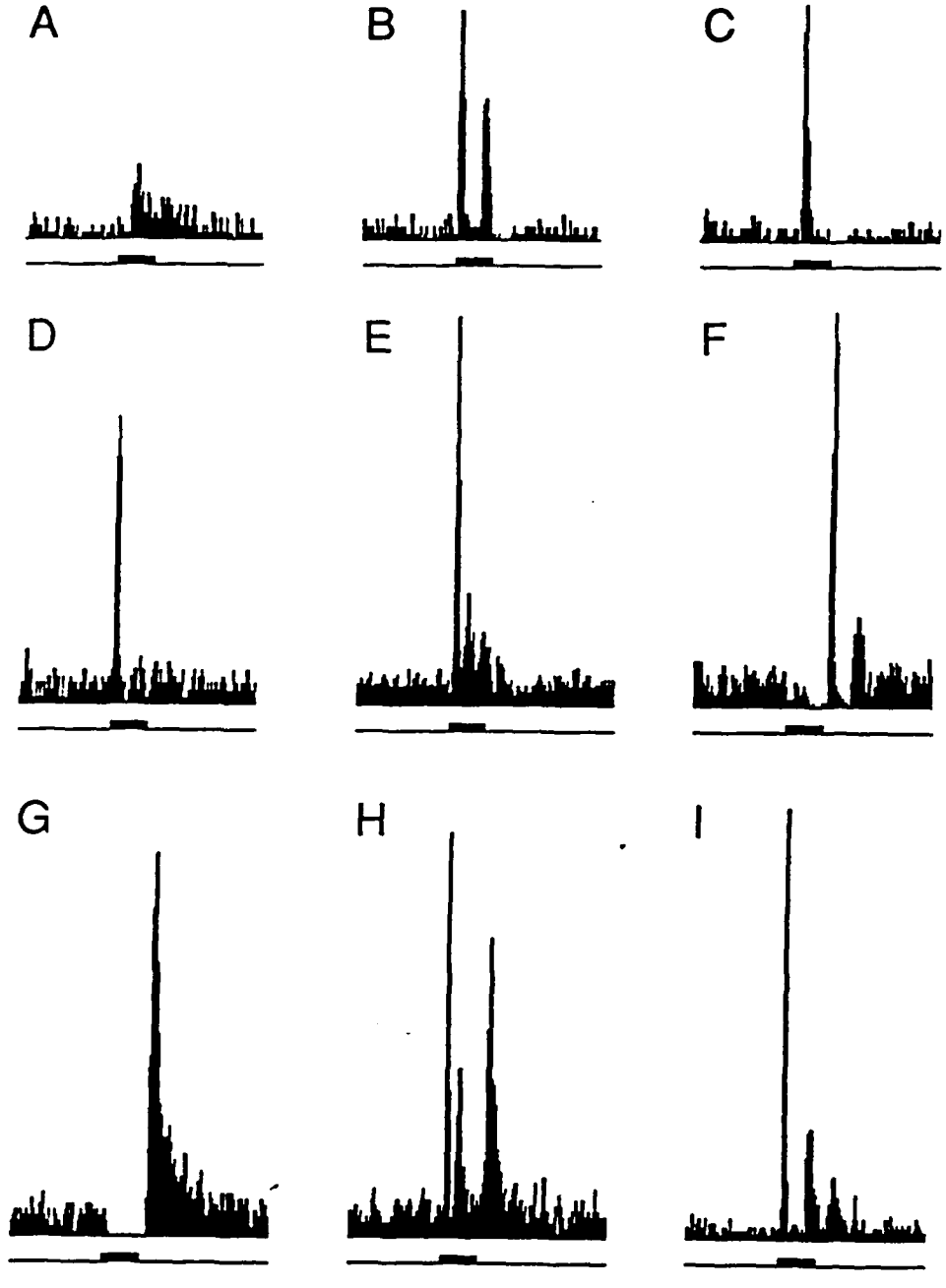

Figure 1. Examples of Poststimulus Histograms

Nine examples of poststimulus histograms (PSTH) which exhibit various response components, as explained in the text. The tone duration (horizontal bar below PSTH) was 200 msec; (vertical scaling is not relevant). Each PSTH is the sum of 20 repetitions of the tone.

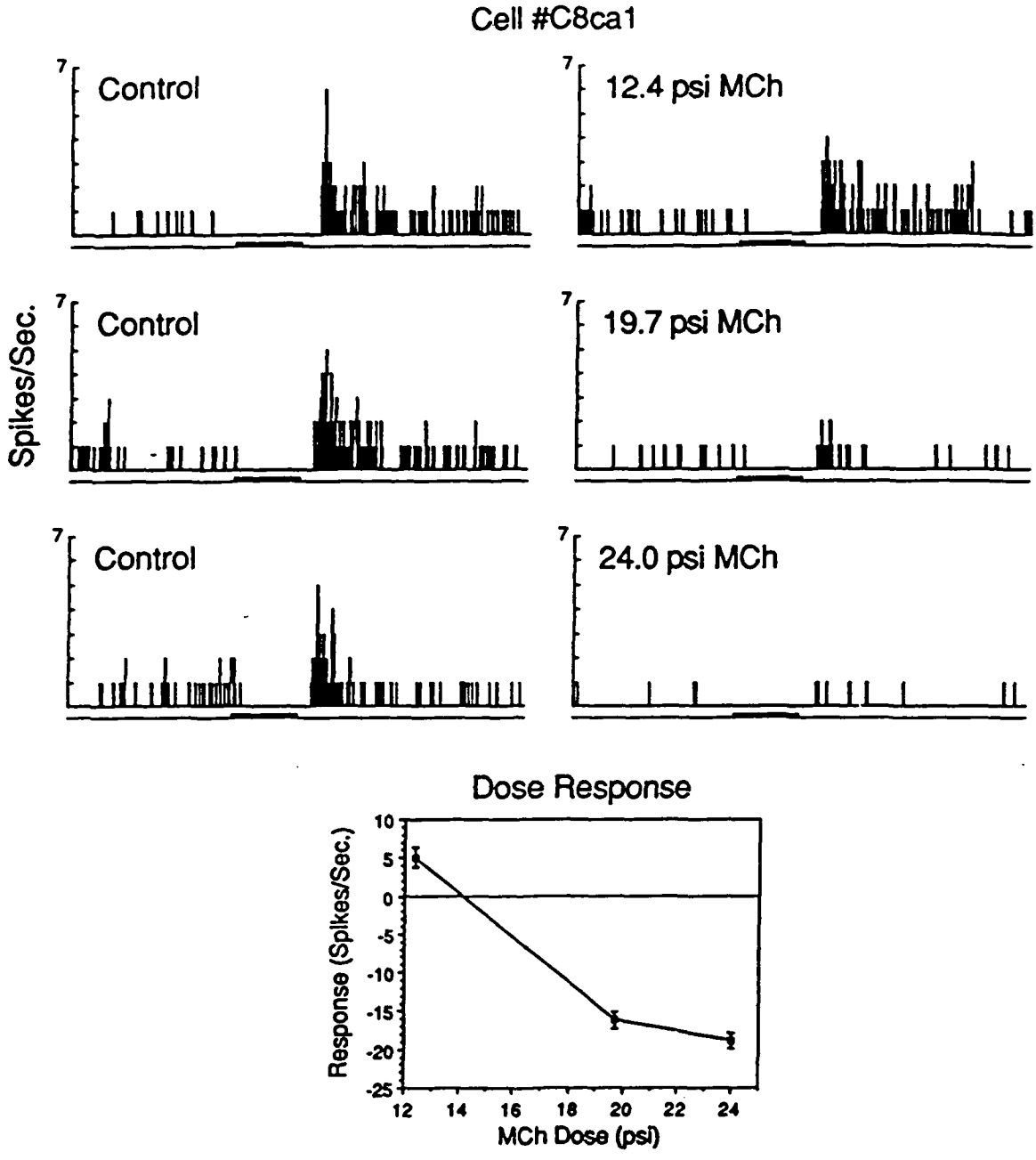

Figure 2. Dose-Response Function for Acetyl-Beta-Methacholine

Top: PSTH during application of MCh (12.4, 19.7 and 24.0 psi) and control periods immediately preceding each application. Note that presentation of the tone (initial upward deflection of marker beneath PSTH), which was 2.5 kHz, 70 db, caused suppression of background discharges and a pronounced "off" response. MCh had no effect on this suppression but did reduce the "off" response in a dose-dependent manner. Bottom: Quantification of the dose-response function. The "response" was determined by subtracting the mean number of discharges in the "off" response during the immediately preceding control period from the "off" response during application of MCh. Vertical error bars indicate the standard deviation (S.D).

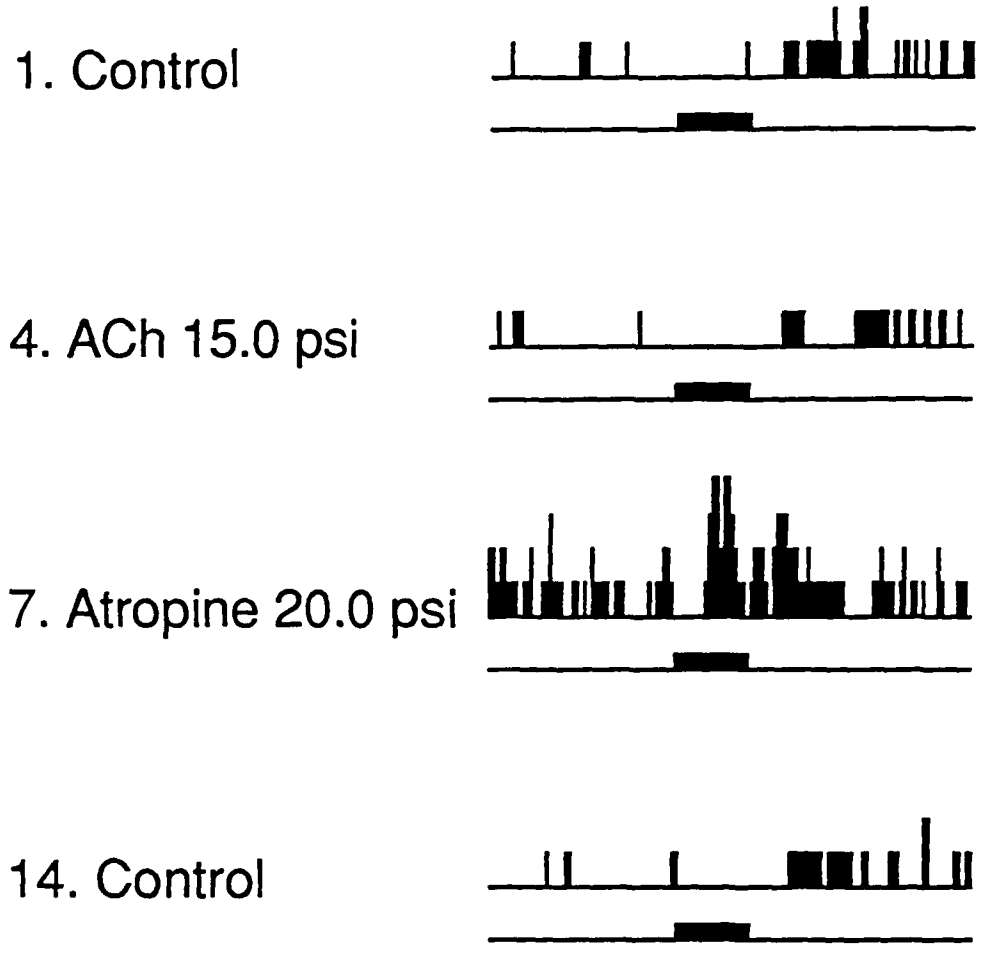

Figure 3. Poststimulus Histograms. Acetylcholine and Atropine

PSTH for cell T36AP, before (#1,"Control") and during application of ACh (15.0 psi), atropine (20.0 psi) and following drug application (#14, "Control"). As in all cases, drugs were applied starting 10 sec before the start of tone presentation and tones were presented at the rate of 1/sec during 20 repetitions of the tone. The interval between the end of one period and the beginning of the next period of stimulation or drug application was 60-90 sec. Tone duration, as in all cases, was 200 msec (marker below PSTH). For this cell, the tone was 18.5 kHz, 75 db. Note that atropine increased background discharges and evoked responses, in contrast to ACh. For quantification of complete data for this neuron, see Figures 4 and 5. The PSTH shown here correspond to sets 1 (first Control), 3 (first application of high dose of ACh, 15.0 psi), 7 (first application of atropine, 20.0 psi) and set 14, the final Control.

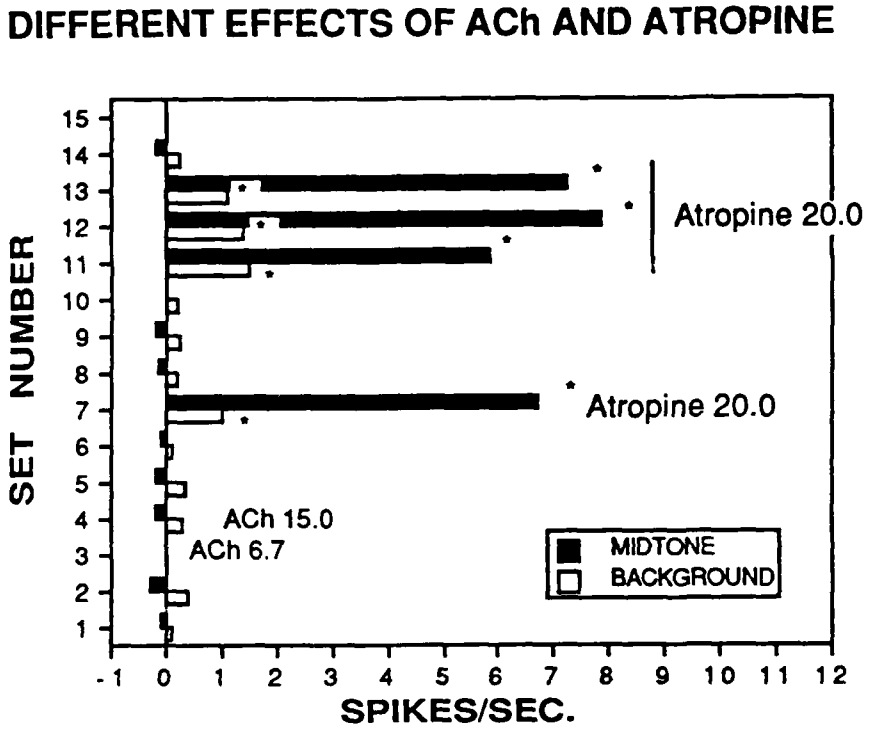

Figure 4. Summary of Discharges for Background and Evoked Response to Tone for Acetylcholine and Atropine.

Atropine facilitated background discharges and the midtone resonses of this neuron. In contrast, ACh has no effect. All atropine data were significantly greater than control data (t test for repeated measures, $p < 0.05$). In this and figures 5,6,8,9, and 10, drugs and doses are shown for stimlulus sets during which they were applied while sets designated only by set number are control periods. Statistical significance at $p<0.05$ is indicated by an asterisk (*).

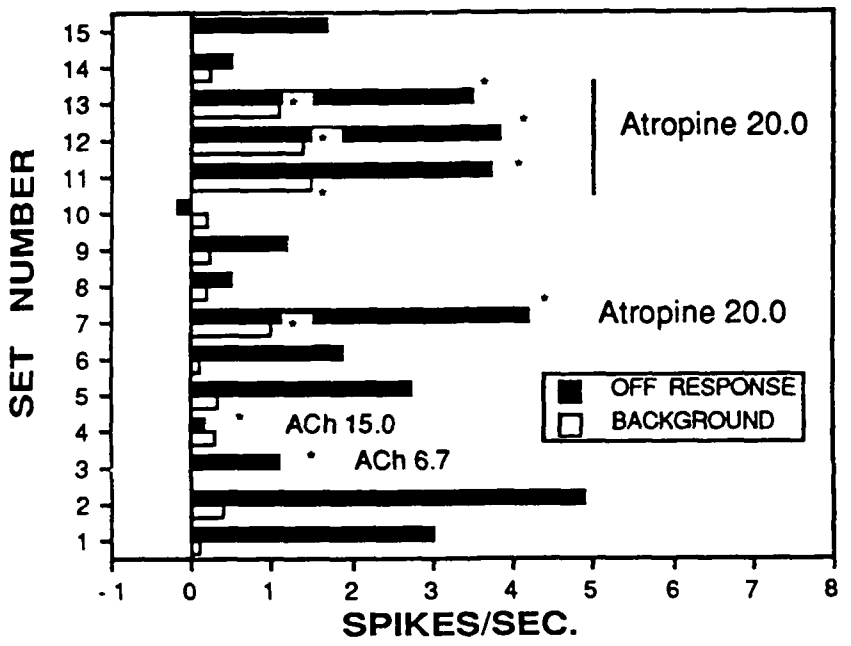

Figure 5. Summary of Background Discharges and Off Response to Tone for Acetylcholine and Atropine.

Data for the same cell as in Figures 3 and 4, showing the effects of ACh and atropine on background and "off" responses. The background data are the same as given in Figure 4 and are included here for convenience. Note that ACh showed a dose-dependent suppression of the "off" response whereas atropine facilited this response. Compared to preceding control periods, all effects were statistically significant (t test for repeated means, $p<0.05$).

Direct antagonistic effects were also observed. Figure 6 presents a summary for the effects of MCh and atropine on background activity. MCh alone produced a significant increase in discharges ("MCh 19.0"), while atropine had no effect ("Atropine 8.5"). However, application of atropine in the presence of MCh blocked the facilitating effects of this cholinergic agonist (sets 6 and 7). When atropine was removed, the facilitating effects of MCh were prominent and immediately evident (set 8). Note also the reversibility of the effect, as seen by complete recovery during the following control period (set 9).

Antagonistic effects were also obtained for evoked activity. Figure 7 presents PSTH for a cell which had a prominent "on" response to tone and a smaller mid-tone response. MCh alone significantly reduced both responses (#3) which showed partial recovery in the subsequent control period (#4). Following total recovery, atropine alone had no significant effect (#5). However, the effects of MCh were blocked by simultaneous application of atropine (compare sets 3 and 7). Quantitative analyses of these effects are shown in Figures 8 and 9. These figures show the clear antagonistic effects of atropine on MCh (compare sets 3 and 7 in each figure). Note the relative selectivity of the MCh effects on evoked activity compared to its negligible effects on background activity.

Direct antagonisms were not limited to atropine. Figure 10 presents a summary of scopolamine antagonism of MCh effects on tone-evoked response. MCh alone depressed the response ("MCh 11.5"); this effect was greater for a higher dose ("MCh 18.0"). Scopolamine alone had no effect ("Scopol. 18.0"). However, scopolamine applied in the presence of MCh blocked the latter's suppression effects (see set 8). In fact, the effects of scopolamine with the high dose of MCh yielded a tone-evoked response which was the same as that occurring during the low dose of MCh alone (compare sets 2 and 8).

4. Conclusions

During this first year of the project, we have found that a considerable proportion of neurons in the auditory cortex are sensitive to cholinergic agents. Further, cholinergic mechanisms appear to involve muscarinic receptors as evidenced by the effects of the muscarinic agonist beta-methacholine. The effects of both ACh and MCh can be blocked by cholinergic antagonists, specifically atropine and scopolamine. The importance of exhaustive and quantitative analysis of both background and all components of the pattern of evoked activity is evident in the findings that a given cholinergic agent often has selective effects on these aspects of neuronal activity. Therefore, an adequate understanding of cholinergic mechanisms will not be possible by merely confining analyses to general rate measures or by summary statements which fail to account for these critical details.

Our data to date are consistent with the view that neurons are embedded within a mosaic of neurochemical influences and that their discharges reflect various influences which can be mediated by different transmitter mechanisms. An example is the relative independence of interactions between cholinergic agents and the "on" and "off" responses. Actually, the fact that cholinergic agents can have selective effects on components of the pattern of discharges evoked by sound may provide an entry point into understanding the way in which they are involved in the coding of information in the brain.

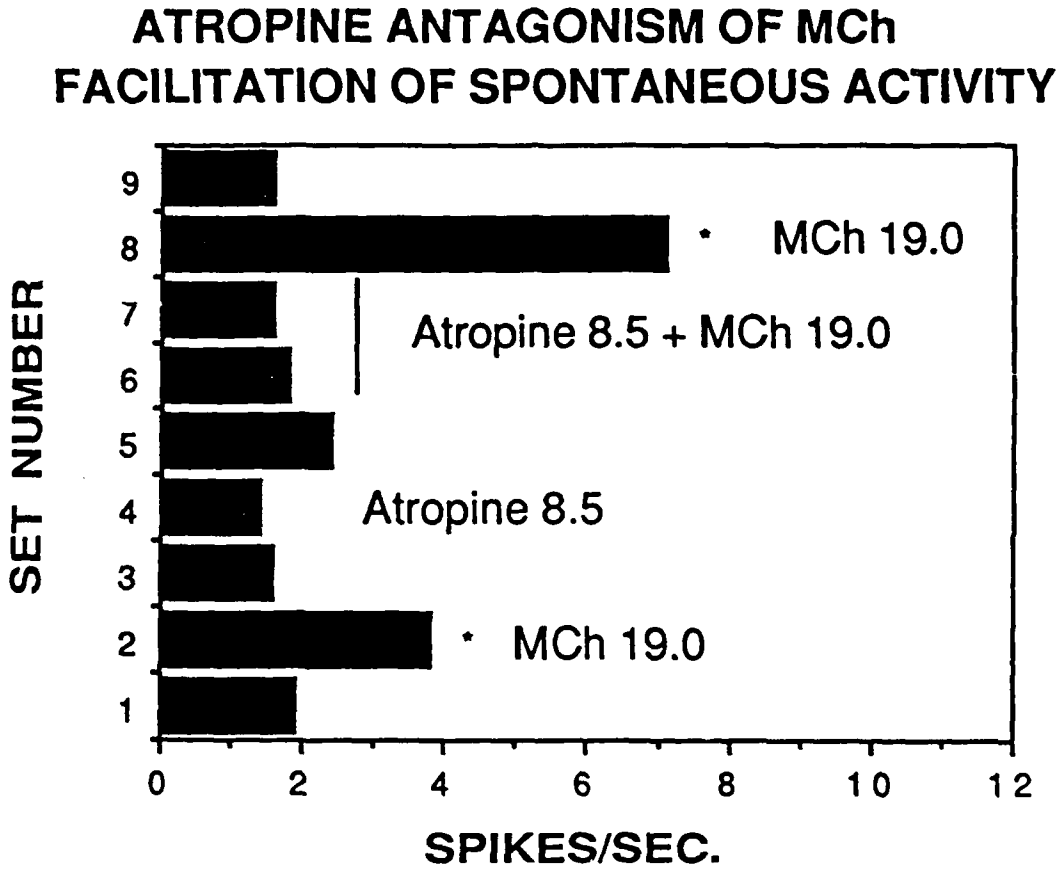

Figure 6. Summary of Antagonistic Effects of Acetyl Beta- Methacholine And Atropine Background Activity.

Data from a cell in which MCh (19.0 psi) facilitated background discharges (set 2) whereas atropine (8.5 psi) blocked this facilitation (sets 6 and 7). Note that removal of atropine application in the presence of MCh resulted in a large facilitation by MCh alone (set 8). Atropine by itself had no detectable effect on background discharges (set 4). The effects of MCh alone were statistically signigicant ($p<0.05$, t test for related samples) compared to preceding control periods while atropine and MCh together did not significantly alter background discharges ($p<0.05$, t test for related samples).

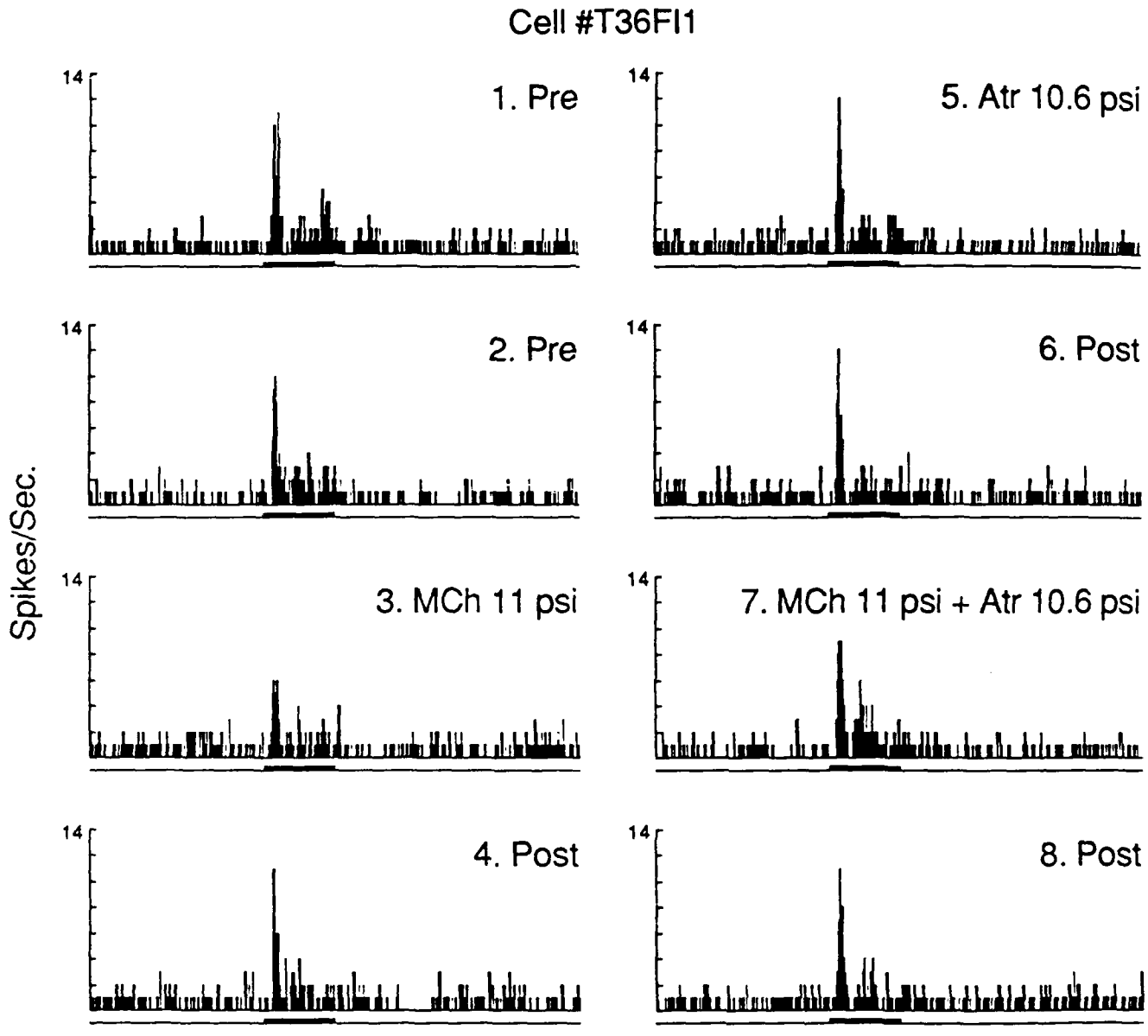

Figure 7. Poststimulus Histograms. Acetyl Beta-Methacholine and Atropine

PSTH for cell T36F. Histograms correspond to the 8 sets for which quantitative summaries are presented in Figures 8 and 9. The numbers for each PSTH correspond to the set numbers in the experiment, and the set numbers in Figures 8 and 9. "Pre" and "Post" refer to control periods during which time tone sets were presented alone. The intervals between sets were 1.0-2.0 min. The tone was 12.0 kHz, 70db, presented for 200 msec., 20 repetitions. The ordinate shows the total number of spikes per 2 msec bin. The horizontal bar beneath each PSTH represents the presentation of the tone (200 msec.).

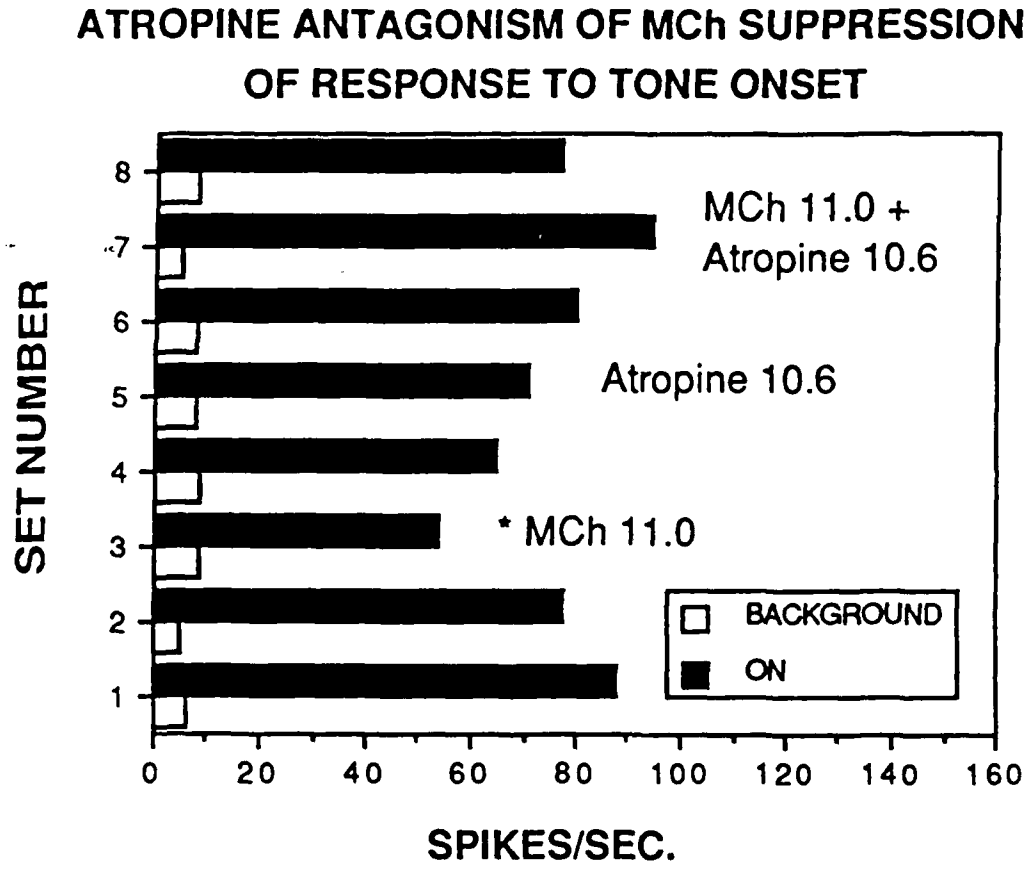

Figure 8. Summary of Effects of Acetyl Beta-Methacholine and Atropine on Tone Onset Response.

Background and evokes discharges for the "on" response. MCh attenuated the "on" response, without affecting background activity (set #3). Atropine, which by itself was without detectable effect (set #5) blocked the effects of MCh (set #7).

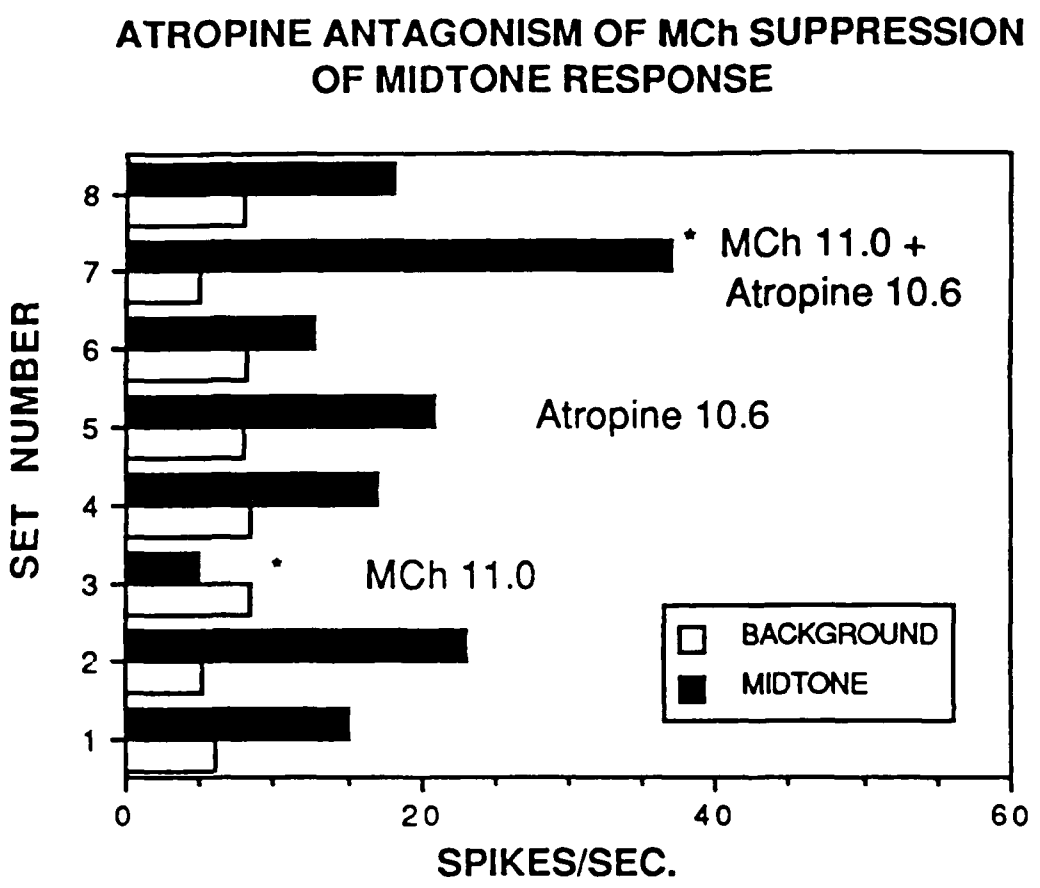

Figure 9. Summary of Effects of Acetyl Beta-Methacholine and Atropine on Midtone Response.

Background and evoked activity for midtone response for cell T36F. Background activity is the same as shown in Figure 8 and is given here for ease of comparisons. MCh attentuated the midtone response (set 3). Atropine antagonized this muscarinic effect (set 7), actually producing a slight facilitation, while having no effect by itself on this response (set 5).

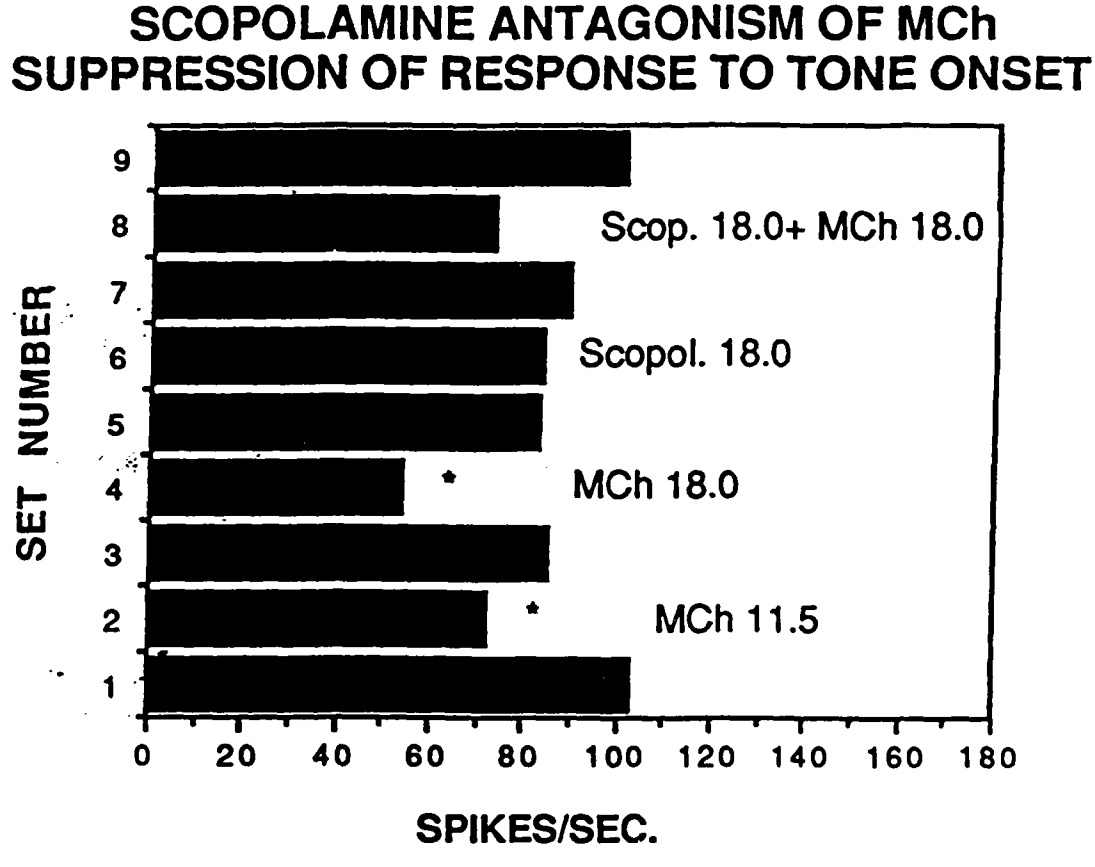

Figure 10. Summary of Antagonistic Effects of Acetyl Beta-Methacholine and Scopolamine.

Data for cell C9AA1. Depicted are the magnitude of "on" responses for the complete 9 sets of data for this neuron. MCh (11.5 psi) produced a partial suppression of the "on" response that increased with increasing dose (sets and 2 and 4) compared to the preceding control periods (sets 1 and 3, respectively) ($p<0.05$, t test for related samples). Scopolamine, which itself did not alter the "on" response (set 6), blocked the effects of MCh (set 8).

B. Computer-based Waveform Sorting of Single Unit Discharges

1. Introduction

As explained in section III.B., a major goal of this project is to obtain data from several single units simultaneously during the local microapplication of one or more cholinergic agents. Furthermore, these recordings must be stable during a prolonged period of behavioral training and the acquisition of the behavioral pupillary dilation conditioned response. As summarized below (section IV.C.), we have optimized the rate of learning using rewarding hypothalamic stimulation. Even so, a complete training session may require 1-3 h, plus time to determine the frequency receptive fields (e.g., "tuning curves") of neurons. Our choice of a strategy based upon recording from several single units simultaneously via one electrode is mandated by the goals of this project. As pointed out above, all of our research on this important technique has had to begin at the beginning as there are no reliable, suitable techniques which could be transferred to this laboratory, because of the absence of statistical confidence limits for waveform selection (64).

2. Criteria

Waveform sorting of discharges from single neurons has a long history which need not be reviewed here. Suffice it to say that there have been three major lines of approach: 1) multiple amplitude/time "window" detectors, 2) special purpose computers, and 3) general purpose digital computers. Each has its advantages and drawbacks. In order to evaluate these, it is first necessary to specify the criteria which must be met for a specific application.

For the purposes of this project, there are both general and specific criteria. The general criteria have, in our view, wide applicability; the specific criteria are dictated by the goals of this project, but could be equally appropriate to a class of similar projects.

The general criteria are as follows:

1. The method must have a very high degree of accuracy.

2. Because any method will involve some error, it must be possible to estimate accurately the Type I and II errors.

3. The method should be easy to use because most of an experimenter's attention must be devoted to the protocol and to other technical matters.

4. The method should either include or provide for an adaptive form of waveform sorting in order to allow for the "tracking" of the same waveforms should recording conditions change, e.g., due to small but significant movements of the subject.

The specific criteria for the current research are as follows:

1. Waveform sorting must be in real time so that the effects of pharmacological agents on each cell can be determined prior to the application of another dose or agent.

2. Waveform sorting should not require inordinate amounts of storage because of limits in equipment and because of the expense and additional time involved, but should include storage of relevant data in order to allow for post-experiment quality and reliability checks.

Applying these criteria to the three general approaches listed above, we concluded that multiple amplitude/time windows and special purpose computers would not be good choices. The first fails to meet the general criteria of accuracy, error estimation, and ease and the specific criterion of storage. Special purpose computers may or may not attain the accuracy criterion, but they appear not to allow for error estimation, and they require constant "knob twiddling." Furthermore, they must in general be used in conjunction with a digital general computer to handle the adaptive storage criterion.

The use of general digital computation does not guarantee satisfaction of all the criteria. The major consideration is, of course, the algorithm selected or invented and its implementation. For example, principal components analysis may be highly accurate, but it cannot run in real time. The demand for accuracy includes not missing any discharges; a reasonable goal for extant technologies is the ability to acquire and sort 500 spikes per second for an indefinite period. In the following section, we discuss the methods which we have tested and explain the bases for our selection of one particular algorithm.

3. Methods

a. Introduction:

We have evaluated in detail three algorithms: two were provided with the cooperation of their developers, and we are extremely grateful for their assistance and generosity. The first algorithm is that of Orban and d'Hollander (65). This will be referred to as "OdH." The second was provided by George Gerstein of the University of Pennsylvania School of Medicine (personal communication). Dr. Gerstein is the acknowledged premier scientist in this field of waveform sorting. His algorithm is referred to as "GG." The third method was developed in this laboratory as an extension of the use of simple features which have been in the general domain for years; although it is difficult to assign proper credit, we have been influenced strongly by van der Molen, et al. (66). We refer to this method, by the initials of our programmer, Dr. Michael Cassady, as "MC." All three algorithms can sort in real time. In fact, they all meet the criteria reasonably well, or could do so with minor modifications. While they are all superior to most other methods, particularly for the present application, there is still ample reason for selecting among them.

b. OdH Algorithm:

Thirty two samples of a waveform, detected external to the computer by a voltage detector, are digitized during a "learning" phase. After 100-200 waveforms have been stored, the differences between corresponding samples (n 32) are computed and the sum of the differences is determined. Waveforms are clustered or classified based on various total differences by the experimenter. These "d" values may be thought of as the distances between the mean differences of each cluster in a 32 dimensional space. An easier visualization is to consider each

cluster of waveforms as comprising an astronomical galaxy, with a dense core and outlying satellites. The algorithm should not misclassify the waveforms within the dense core, but the outliers, which can represent a considerable fraction of total waveforms, could be misclassified.

This method appears to make maximum use of all data because it computes differences based upon all 32 samples. However, the samples are not statistically independent. For example, the samples immediately adjacent to the sample at the peak of a waveform are always very close to the peak. Furthermore, samples which occur prior to the start of the actual waveform or after its completion are effectively not promoting sorting and may be a source of error. For example, they may not be truly random values, reflecting the immediately preceding activity, e.g., unit waveforms from cells which are too small to trigger the voltage detector but which are not randomly related to the cell which is firing. A major problem involves determining and setting confidence limits. Although this is statistically feasible if the sampling distribution of the differences is known, in practice we have not pursued the matter deeply, given the other more basic drawbacks. In fairness, I do wish to point out that the method works quite well on sorting waveforms automatically, which an experimenter can discern on a storage oscilloscope.

c. GG Algorithm:

George Gerstein developed the most successful special purpose computer for waveform sorting. Even so, he switched to general purpose digital computers due to their inherent advantages. While the special purpose hardware is in fact able to sort faster, the problems of continuous fine tuning and inherent errors mandated the change.

The GG method starts with a 128-point digitization of an externally detected waveform. Usually, 60-150 waveforms are digitized and are combined into one grand waveform. Each of the 128 points is then scrutinized for the largest variance; this point is selected as the best discriminator among all of the waveforms; it need not be a peak value. By a mathematical formula which we have not received, a second point is selected; this is not a point adjacent to the first, but in the absence of the formula, we cannot specify the rule. This "maximum" variance technique is iterated for up to eight points on the waveforms. In practice, we have found that six points yields asymptotic waveform sorting. Once the points have been determined, a difference value is obtained based on analysis of variance. The experimenter selects the difference value which yields the most stable separation. This, then, constitutes the learning set. As with the OdH technique, a template based on the critical feature points is employed during subsequent data acquisition.

In its present form, the GG method is a forced classification technique. That is, if a new waveform appears, it will be classified with those to which it is most similar. The need for confidence limits is clear here, and they could be developed. This method has the advantage of using the best criteria for separation, without any preconceptions about peaks, etc. Dr. Gerstein is presently using it to advantage.

d. MC Algorithm:

The MC program for sorting waveforms, like the OdH and GG approaches, has two phases: a learning phase in which a template or critical features are determined for each class, and a data acquisition phase, during which the actual experiment is run and waveforms are acquired and sorted. As with the GG program, an externally detected waveform is digitized into 128 values. The method then determines the largest positive and negative values (the peaks) and the time between the peaks with an accuracy of 13 μS. This learning phase uses about 500 spikes, which is advantageous in the next stage. van der Molen et al. (66) emphasized the importance of using these three parameters for spike sorting. The MC enhancement is to compute means and standard deviations for distributions of the negative and positive peaks and the latency interval between them. This provides essential data for determining confidence limits. The experimenter selects the boundaries of the distributions, which are generally Gaussian. This "first cut" eliminates obvious infrequent values which, even if generated by individual cells, would occur too infrequently to permit statistical evaluation. In practice, this is not a limitation because several cells with adequate probabilities of discharge are still present.

The next steps are quite novel. The total number of logically possible waveform classes is determined by the product of the number of amplitude and latency distributions. The exact probability of each waveform in each distribution is determined, based on a stored table of the normal distribution. For each waveform, its latency and amplitude probabilities are multiplied, yielding a probability for each waveform within each possible amplitude-frequency class. The waveform is then assigned to the class for which its conjoint amplitude-latency probability is highest. This also provides the exact probability of that waveform for the selected class. For example, a waveform which is at the mean of the class to which it has been assigned has a probability of 1.0 that it lies within this distribution. All other waveforms within that class have lower probabilities, but all of these probabilities are greater than the probability that the waveform belongs to another class. The experimenter then sets confidence limits, e.g., 0.05, 0.01, etc. Thus, waveforms in the "tails" of the distribution can be eliminated as desired.

During the second data acquisition phase, each waveform will be assigned to a class based on its amplitude, latency between peaks, and polarity of the first peak. Artifacts or new waveforms will be rejected unless they fortuitously meet the criteria for selection. The percentage of unclassified waveforms is also computed to provide an estimate of the number of new waveforms, if any, which appear. Should this percentage become unacceptable, a new learning phase can be run and the process repeated. After moving the electrode to another position, it is essential to repeat the learning phase because the algorithm is based upon the actual distributions of amplitude/latency parameters rather than on some universal values which would be inappropriate.

The MC algorithm makes use of the peaks and interval between peaks of a waveform. These three features are in common use, even with some special devices. However, it transcends previous uses by its ability to set confidence limits in an easy and straightforward manner. Although it does not make use of more than three features, these are in practice sufficient because virtually all other sample points are statistically related to these three values. This method cannot discriminate between waveforms with the same peaks and time-between-peaks

which have different features on the ascending or descending parts of the waveform. However, cells with such "notches" are generally rejected because they may be injured.

An example of a test of the MC algorithm is presented in Figure 11. In this case, four synthetic "waveforms" were intermixed in a spike train. The type and number of each waveform were known in advance. The picture at the top shows an oscilloscope record at the test inputs to the program. The histograms below show that four distinct time intervals between peaks were detected. Also, four amplitudes were detected. These correspond to the fact that the test stimuli each had a different duration and amplitude. The waveforms at the bottom are the average waveforms (plus and minus one standard deviation) which were sorted by the program. They correspond to the inputs, demonstrating the efficacy of the MC algorithm.

Figure 12 shows an example of waveform sorting of records from two cells recorded simultaneously in the auditory cortex. As in the case of synthetic waveform, the MC algorithm correctly sorted and classified the real waveforms. Final tests with more complex data are under way.

A schematic diagram of the entire system for controlling the experiment and performing real time on-line waveform sorting is presented in Figure 13, as it will appear in final form. It is a multiprocessor system, with each processor devoted to a particular task. Following detection of a waveform by a voltage window, a PDP 11/03 computer, which controls delivery of calibrated acoustic stimuli and is in overall control of the experiment, digitizes the waveform and sends the 128 values to the central processing unit (CPU) of a PDP 11/73 minicomputer over a special transfer line; receipt of the data is checked at this time. The waveforms are classified on the basis of the immediately preceding learning phase for the cells in question. Three Mackintosh computers are used in place of graphics terminals because they are faster and provide high resolution graphics for little more than the cost of a terminal, they can store the results of analyses and provide additional analyses without interrupting the experiment, and they provide for immediate hard copy of waveforms and other relevant graphics (e.g., tuning curves) without the need to write graphics programs.

4. Conclusions

All three methods have been tested on the same known data sets. These sets consisted of synthesized waveforms in which various parameters were systematically altered. They were also compared on data sets from actual recordings. The general order of accuracy was MC, OdH, and GG. This ordering may reflect the lack of confidence limits in the GG program at this time, so the comparison is not fair on an absolute basis. However, we are not concerned with the best possible ultimate methodology, but only with the means to accomplish the goals of this project, given limitations of time and resources. Accordingly, we have selected the MC algorithm. It is currently being incorporated into the rather complex programs which now run the experiments.

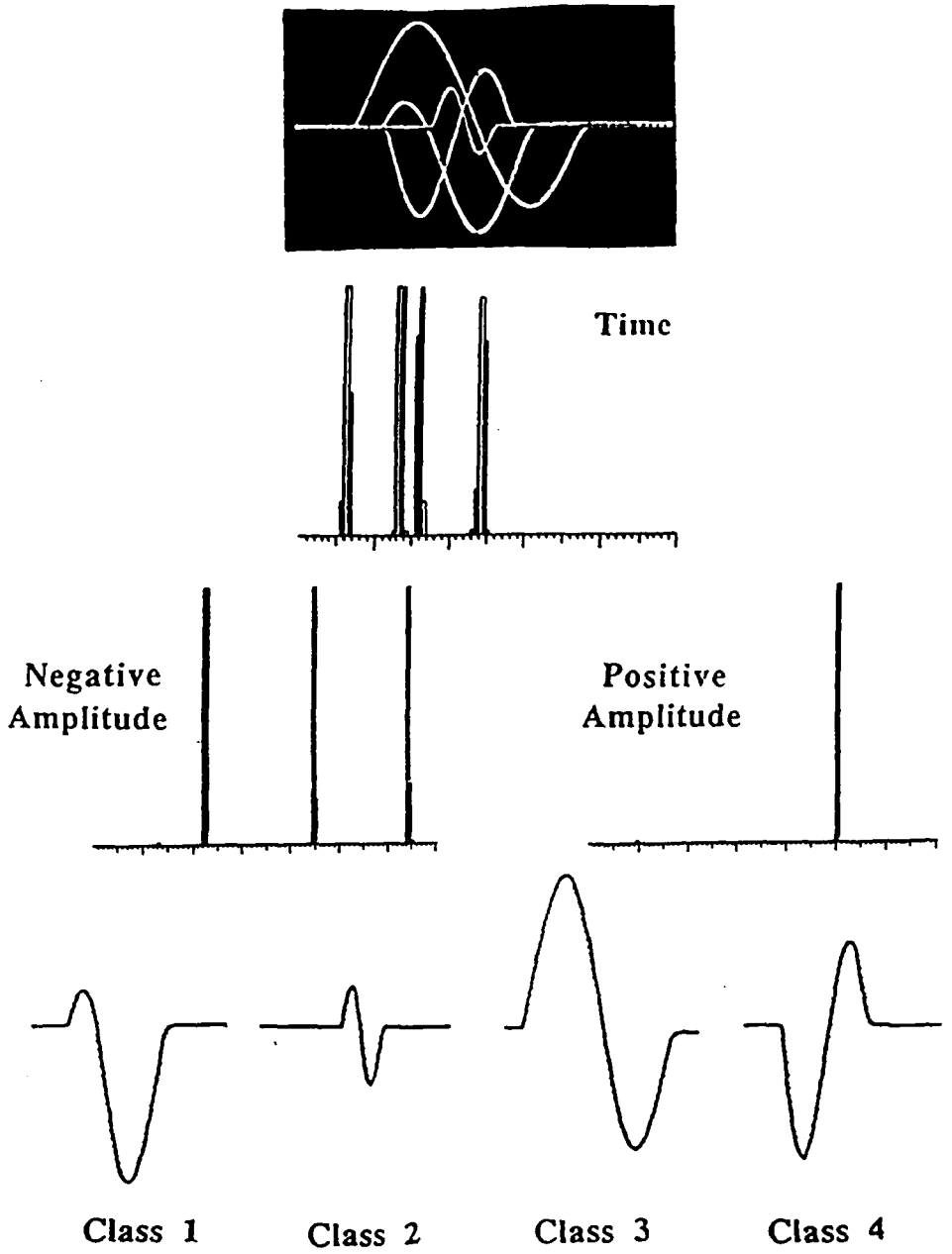

Figure 11. Test Data and Waveform Sorting for MC Algorithm.

Top: Oscilloscope traces of four artificial waveforms used to test waveform sorting algorithms. The wave forms differed in duration (time between first and second peaks) and peak-peak amplitude. Also, three waveforms had a negative second peak whereas one had a positive second peak. Middle: Distributions of the time between peaks, the amplitudes of the negative (downward) wave and the amplitudes of the positive (upward wave), of approximately 50 waveforms of each type. Note that the time and amplitude/polarity distributions produced by the program show the four different durations and amplitude/polarities of the input signals. Amplitudes are arbitrary A/D units, hence calibrations are not given to avoid confusion. The time scale is in MS (largest tic marks). On time and amplitude scales, increasing values are to the right. Bottom: The results of using the MC algorithm; note that it successfully sorted the four artificial waveforms that were input. The sorting was based on combinations of time and amplitude distributions.

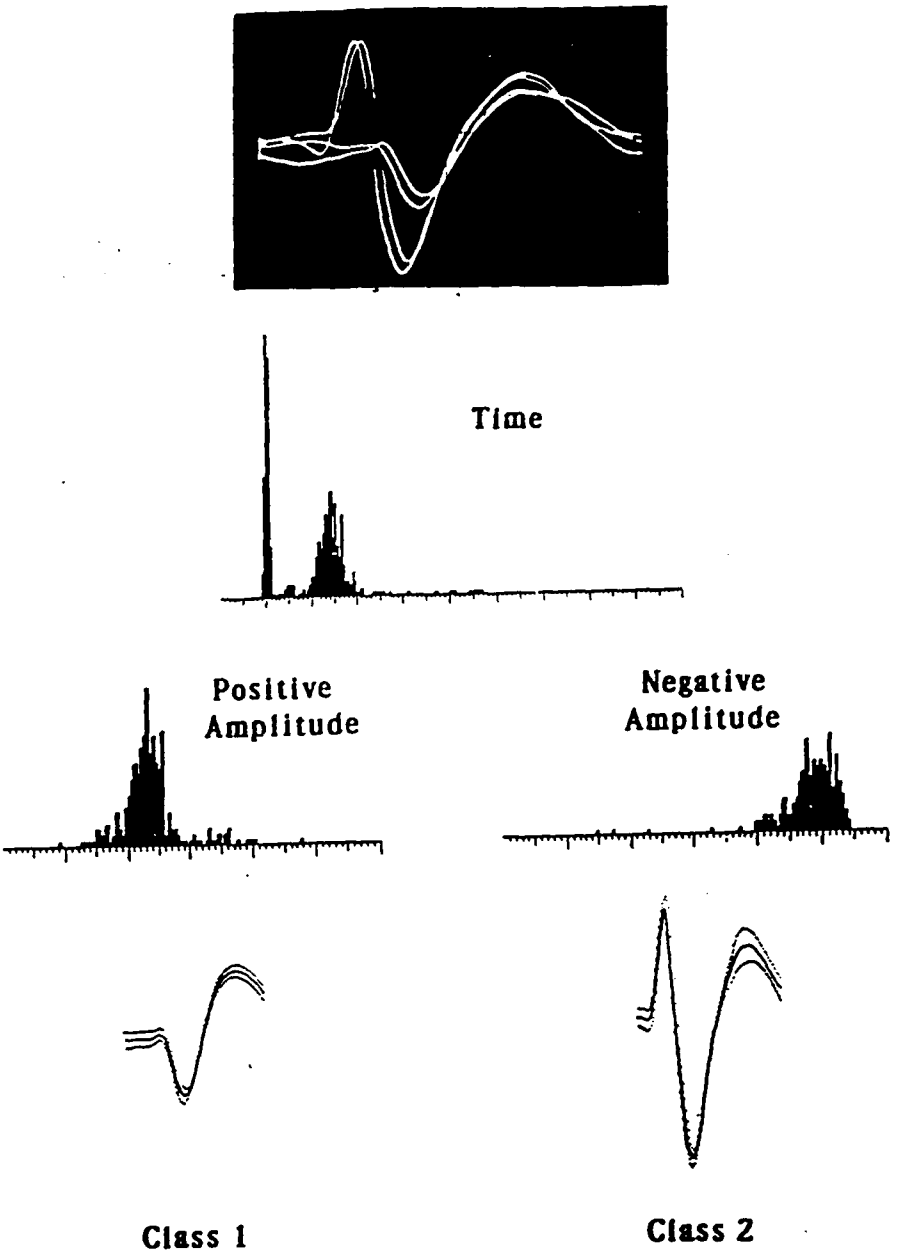

Figure 12. Neuronal Data and Waveform Sorting for MC Algorithm

Top: Oscilloscope trace of neuronal waveforms obtained from auditory cortex of the cat. Middle: Time and amplitude distributions for 500 spikes admitted to the MC algorithm "learning phase". Note the bimodal distribution of times (i.e., spikes widths) and the unimodal amplitude distributions. These graphs are in units of time (1.0 ms/largest tic mark) and amplitude (100 microvolts/largest tic mark). Bottom: Average unit waveforms (with 1 S.D.); compare with the sample of input waves (top). Note that the MC algorithm successfully recovered these waveforms. Note also the small variance in the waveforms.

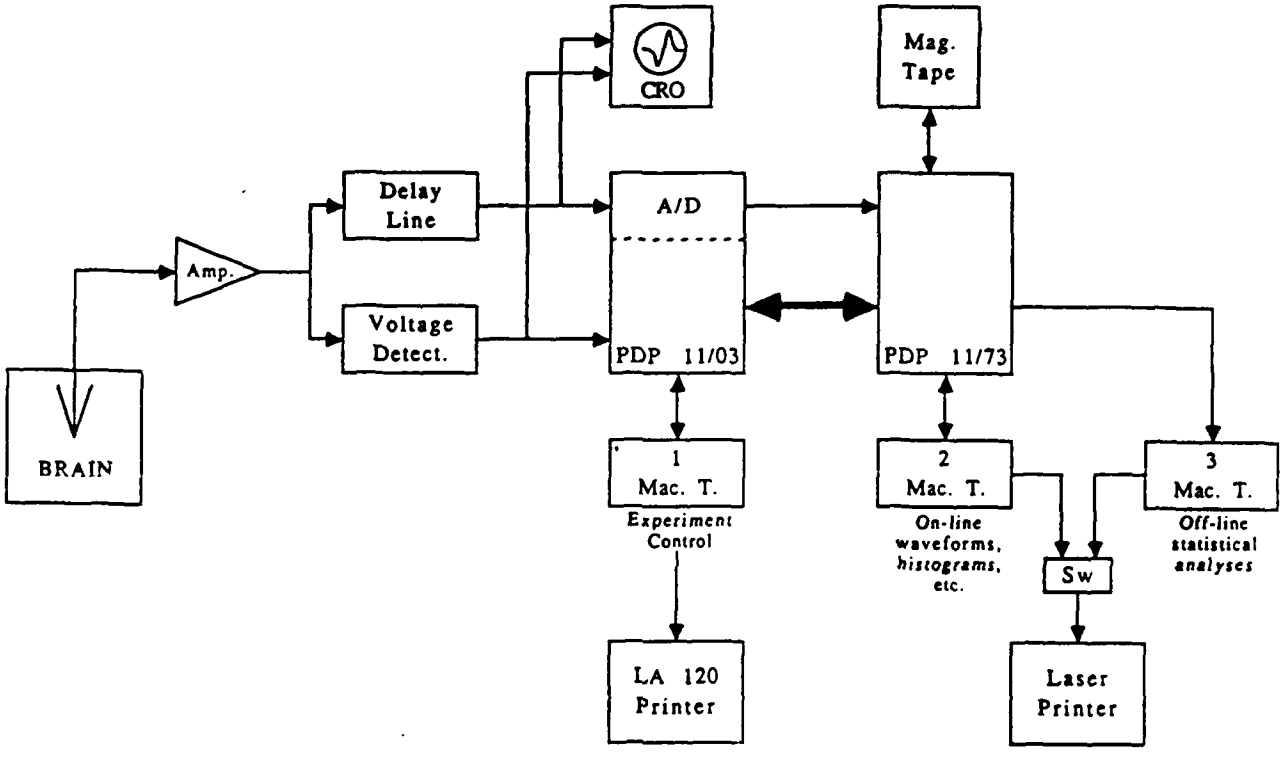

Figure 13. Schematic Diagram of Experimental Control and Waveform Sorting System.

The relation among the hardware components of the experimental control and waveform delivery system.

C. Behavioral Learning: Hypothalamic Self-Stimulation and Classical Conditioning

1. Introduction

Recent single unit recordings from the cat primary auditory cortex (AI) have demonstrated that cell-tuning properties are significantly affected by learning (63). In these experiments, cats were prepared under pharmacologically maintained neuromuscular blockade. However, the potential complications of the use of a synthetic curare derivative, gallamine triethiodide, or similar agents in the study of cholinergic mechanisms require that another suitable preparation be employed. We had planned to use liver extract delivered directly to the oral cavity as a reward. However, anticipatory jaw movements during conditioning would preclude constancy of acoustic stimuli at the cochlea. Consequently, we have developed an undrugged preparation in which rapid learning develops using rewarding hypothalamic stimulation. The conditioning paradigm and method of restraint which will enable us to record from such a preparation are described below.

2. Methods

a. Surgical Preparation:

Healthy male adult cats, 3.5-5.7 kg, were anesthetized with sodium pentobarbital (35 mg/kg, IP), intubated, and placed in a stereotaxic frame. The skull was exposed and cleared of muscle and connective tissue. Through two small holes drilled bilaterally, bipolar stainless steel stimulating electrodes (Frederick Haer and Co.) were lowered to the posterior hypothalamus at stereotaxic coordinates A-P 10^{o}, D-V-3.5, and M-L 1.2, (67). Wilkinson and Peele (68) demonstrated that electrodes placed at these coordinates elicited self-stimulation. Cell activity was measured every 2 mm while lowering the electrodes to generate voltage profiles useful in confirming electrode position. The electrodes were fixed in place with dental acrylic secured to the skull with stainless steel screws. The acrylic was molded into a pedestal with two metal spacer nuts which allowed for nontraumatic immobilization of the head in subsequent training sessions. A warm heating pad placed under the cat maintained body temperature during surgery. Terramycin ophthalmic ointment prevented corneal drying, and antibiotics were applied to exposed skin surfaces. Cats were maintained in an incubator during postoperative recovery.

b. Nonstressful Animal Restraint:

For experimental sessions, an awake cat was placed in a plywood box lined with soft foam rubber. Inside dimensions of the box measured 6" high, 6" wide, and 12-18" long, adjusted with a sliding back. A sloping front provides ample space for extending the forelimbs. Movement of the head is restricted by two opposing U-shaped, padded plates which are affixed to slides so that the opening for the neck can be adjusted according to the size of the animal without pressure on the neck or head. A soft pad made of foam rubber is placed under the chin to provide a convenient head support. Thus, the cat does not have to hold its head. The general posture assumed is that of quiet resting or sleeping except that the head is not lower than the body.

No adaptation to the box is required, in contrast to previously reported restraining boxes which require pressure on the snout (69), and cats have rested comfortably for several hours with no vocalization, struggling, or other signs of stress.

c. Animal Comfort:

To ensure that cats were comfortable, all contact surfaces were soft and padded. Animals were closely monitored and were released on those few occasions when signs of discomfort were evident. Periods of restraint were not long enough to cause discomfort due to absence of food or full bladders. Significantly, cats so restrained readily purred when petted, and many cats fell asleep while in the box. From all reasonable indicators, the box is a nontraumatic restraint.

d. Hypothalamic Stimulation:

Hypothalamic stimulation was delivered as a 600 ms train of 100 μs pulses at 100 Hz. These parameters were determined empirically to be maximally effective and were modified from those described by Affanni et al.(70). Stimulus intensities ranged between 400 μA and 5000 μA. With correctly positioned electrodes, low intensity stimulation caused short latency pupil dilation. Increasingly higher intensities elicited eye movement, head movement, and licking. Only those currents eliciting simple pupillary dilation were delivered during classical conditioning and self-stimulation sessions. In later learning experiments, stimulus intensity was chosen as that eliciting maximal pad-pressing behavior during self-stimulation trials.

e. Self-Stimulation:

While restrained in the box, a cat was allowed free head movement and could rub against a soft pad suspended near its head. The cat could self-stimulate by pushing this pad, and an underlying switch, to complete a circuit which delivered current to a hypothalamic stimulating electrode. Each pad push delivered a single stimulus train. The baseline frequency of pad pushing was determined at 0 uA, where depressing the switch did not result in brain stimulation. An increase over baseline in hit frequency at intensities greater than zero provided evidence of self-stimulation. Hits were measured at 0 μA as an upward deflection on a Grass model 7 polygraph and at higher intensities as a downward deflection. The effect of stimulus intensity on self-stimulation was determined by measuring the number of pen deflections in a 5-min period for each current tested. Order of presentation was randomized to avoid time effects.

f. Classical Conditioning:

For learning studies, acoustic and hypothalamic stimulation served as the conditioned stimulus (CS) and the unconditioned stimulus (US), respectively. The conditioned response (CR), in the form of pupillary dilation, was monitored with an infrared pupillometer which accurately measures pupil diameter and distinguishes between eye blink, movement of the nictitating membrane, and eye movement (71). To provide stability in measuring pupillary dilation, the cat's head was immobilized by bolting the cranial pedestal to a heavy support. Sound was delivered through a calibrated speaker placed near one ear. Hypothalamic stimulation is described above.

The training paradigm included sensitization, conditioning, discrimination, and discrimination reversal. This method of training controlled for nonassociative effects and demonstrated that associative effects were not restricted to a particular combination of CS and US. Each training phase is described below.

i) Sensitization -- This phase consisted of 15-20 trials each of the positive CS (CS+) and the US, presented in an unpaired fashion at unpredictable intervals with an average density of two per minute. The US did not occur within 10 s of acoustic stimulation. For studies of descrimination between two tones, the tone that would not be paired with the US is designated as the negative "CS" (CS-). Sensitization for discrimination experiments included unpaired presentations of both the CS+ and the CS- (15 to 20 trials each of the CS+, CS- and the US). Sensitization served as the baseline against which subsequent effects of conditioning and discrimination were measured.

ii) Conditioning and Discrimination -- Conditioning trials were initiated without interruption following the last sensitization trial and continued for 20-60 trials. The CS+ and US were always paired, with the US following the CS at stimulus offset. In discrimination experiments, the CS- was presented, but never paired with the US. In all cases, stimulus density was maintained at two per minute.

iii) Discrimination Reversal -- Following the last discrimination trial, training continued with a reversed stimulus paradigm. The tone representing the former CS- was paired with brain stimulation, and the former CS+ was unpaired.

g. Data Analysis:

Pupillometer output was recorded on a Grass model 7 polygraph and measured as millimeters of pen deflection. For each trial, a baseline measurement was subtracted from the peak amplitude to yield a difference score which reflected the pupillary response for that presentation. Baseline was defined as the level of pupillary dilation immediately preceding the presentation of the CS and preceding the US when hypothalamic stimulation was presented alone during sensitization.

Pupillary conditioning reached criterion when the response in each of five consecutive trials was greater than the average of the last five trials of sensitization. Using this paradigm, reaching criterion by chance has a probability of 0.03 (72).

3. Results

a. Self-Stimulation:

Self-stimulation behavior was consistently elicited by weak stimulation of the posterior hypothalamus. Repeated trials within one experimental session demonstrated that cats will significantly increase pad pressing when this behavior leads to brain stimulation (stimulus ON, Fig. 14, $p < 0.001$, t-test for the difference between the means of paired samples). As shown in Figure 14, the frequency of self-stimulation ranged from 26 to 53 hits/5 min. With stimulus OFF, pad pressing frequency never exceeded 8/5 min.

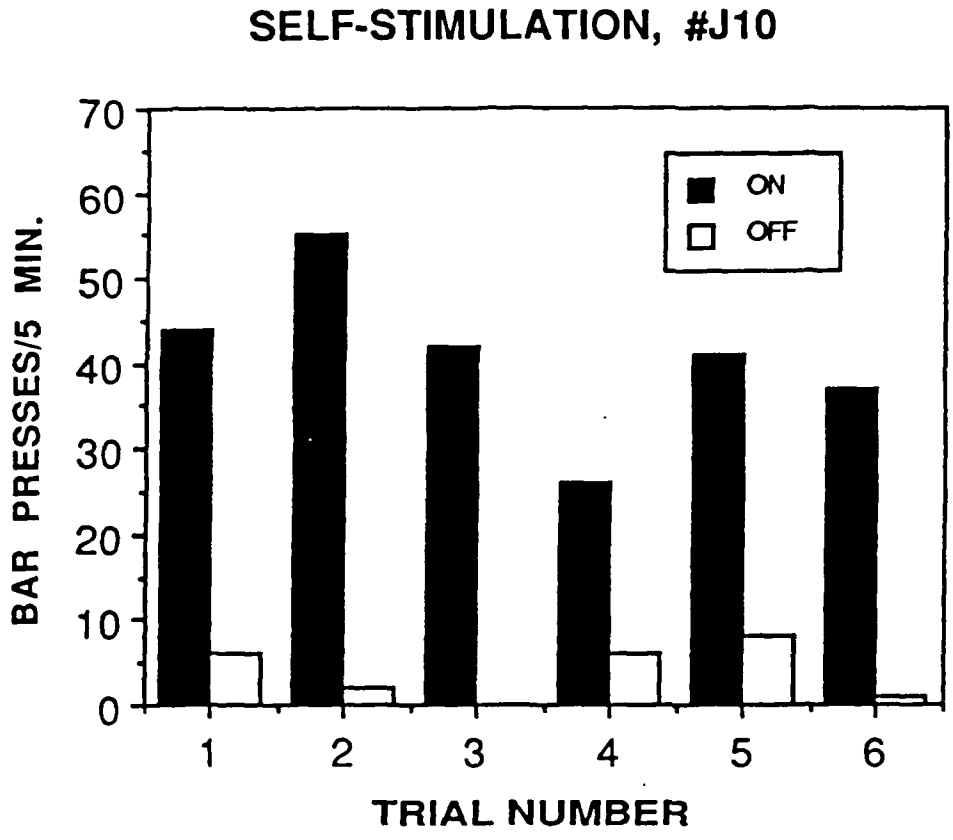

Figure 14. Self Stimulation in the Cat.

The number of bar ("pad") presses for successive periods of 5 min for cat J10 when these responses produced hypothalamic stimulation ("in", closed bars) or no stimulation ("off", open bars). All comparisons are statistically significant ($p<0.01$, t test for related measures) between the two conditions.

Intensity of brain stimulation significantly affects the rate of self-stimulation, as demonstrated by the behavior of cat J11 (Kruskal-Wallis test, $H > X^2(0.001)$, (73) (Fig.15). In this animal, a 400-μA stimulus did not elicit self-stimulation, but appetitive ("rewarding") behavior was clearly evident at 600 μA. The greatest behavioral effect was seen at 1400 μA. Interestingly, a small change from the most effective intensity (1400-1500 μA) resulted in a large decrease in hit frequency, from 34 to 5 presses/5 min. These data suggest an inverted "U" function in the "dose-response" curve for rewarding self-stimulation.

These findings clearly demonstrate that in our learning experiments the US is rewarding, not punishing.

b. Classical Conditioning:

i) Conditioning -- Pupillary responses were similar to those reported in learning studies employing paw shock as the US (63). Briefly, early in sensitization an acoustic stimulus elicits pupillary dilations of small amplitude relative to those recorded in response to the US. In later sensitization trials, acoustically evoked dilation responses are greatly reduced. Hypothalamic stimulation produces large dilations throughout a recording session. During conditioning, the amplitude of acoustically evoked dilations increases rapidly, often approaching in size the US-evoked responses. Pupillary conditioned responses reached criterion in all four cats tested, and rapid learning was achieved in five of six experimental sessions. With rapid learning, mean trials to criterion was 11.80 (SD = 5.36; range, 7-21 trials). One cat learned slowly, not reaching criterion until the 47th trial. If this datum is included in the other five sessions, the mean trials to criterion becomes 17.67, which is still much faster than most other conditioned responses.

The dynamics of pupillary conditioning is illustrated in Figure 16. Each trial block contains five trials. Pupillary dilation decreased very rapidly during sensitization, reaching a minimum by the second trial block (trials 6-10). During conditioning, responses attained criterion by the 10th trial (block 5) and continued to increase in amplitude to the 30th trial (block 9). Unpaired presentation of the CS and US resulted in rapid extinction of the conditioned response; pupillary responses decreased to sensitization levels by the 15th extinction trial (block 12) . It is interesting that the amplitude of pupillary dilation is smaller during extinction than during sensitization. Several explanations are possible: 1) the elevated dilations during sensitization reflect a sensitization effect, 2) a CS presented during extinction (and hence after a history of conditioning) is not equivalent to that same CS presented to a naive animal, or 3) because a US is present during sensitization and not during extinction, the two training phases are not equivalent, and hence differences in pupillary dilation baseline are to be expected.

Subsequent pairing of the CS and US in reconditioning trials illustrates savings (Fig. 16). In contrast to the first conditioning session in which criterion is reached during trials 6-10, pupillary dilation reaches significance during reconditioning within trials 1-5 (block 16). This savings demonstrates that the rate of learning, although rapid, has not reached saturation; learning is possible in the first trial block.

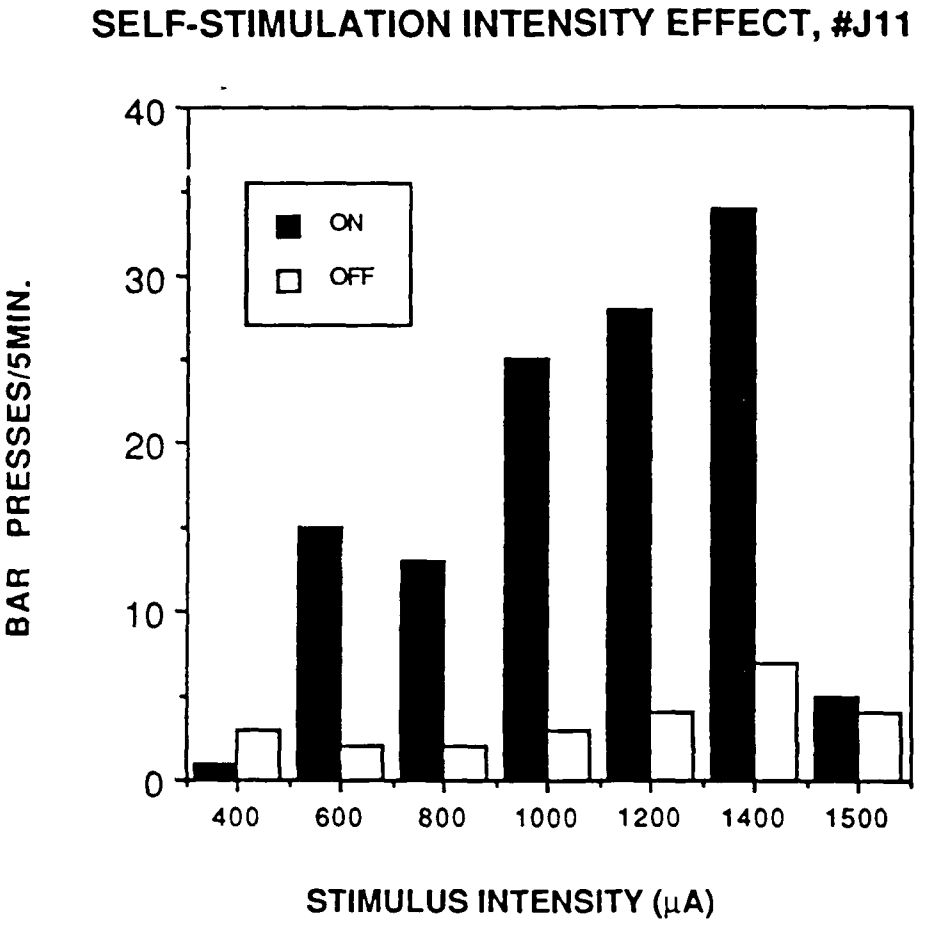

Figure 15. Intensity Function for Self-Stimulation

This graph show that animal J11 produced more bar presses for higher levels of brain stimulation, between 600 and 1400 microamps ("on"). The threshold was above 400 microamps. All comparisons to the paired period of non-stimulation ("off") were significant ($p<0.02$, t test) for the reinforcing range.

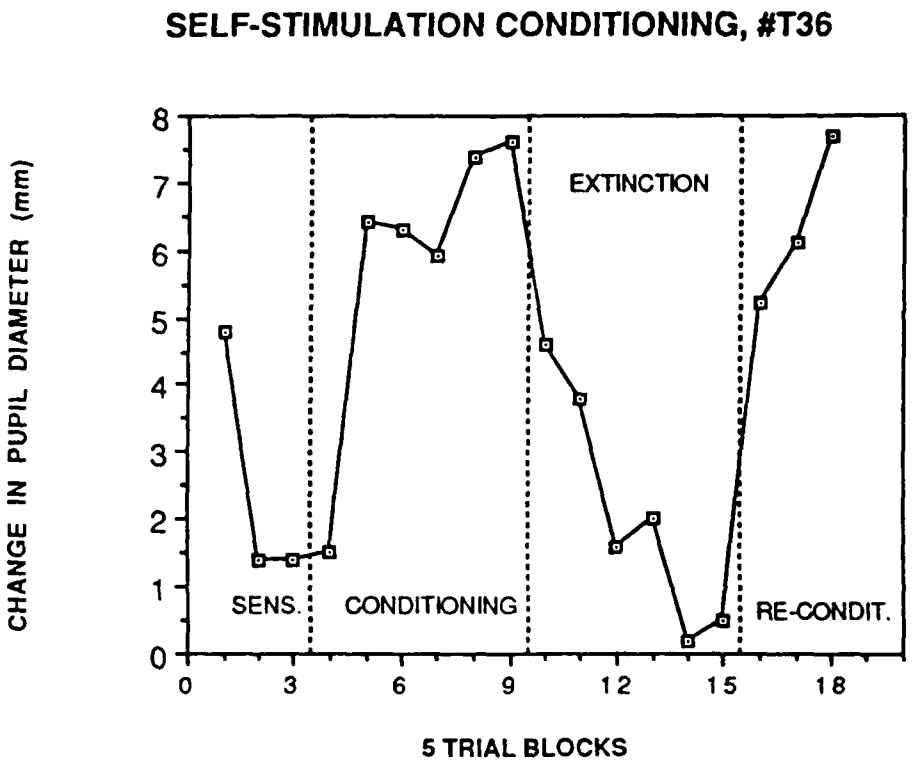

Figure 16. Classical Conditioning with Hypothalamic Stimulation

Change in pupillary diameter is presented for animal T36 during unpaired presentation of the CS and US ("sens"), pairing ("conditioning"), subsequent presentation of the CS alone ("extinction") and a second phase of pairing ("re-condit."). Note the decrease in response during periods in which there was no pairing and the increase in response during periods of pairing. These data also illustrate the rapidly of learning using hypothalamic reinforcing stimulation as the US.

ii) Discrimination and Discrimination Reversal -- Successful discrimination of the CS+ and CS- demonstrates that the CR is specific to the conditioned stimulus and therefore is not a generalized response to all tones. That the CS- could be an adequate conditioned stimulus is proved in discrimination reversal. Figure 17 illustrates that cats are able to perform both discrimination and its reversal with hypothalamic stimulation serving as the US. During the last sensitization trial block in Figure 17, pupillary dilations in response to 2.0 kHz and 7.0 kHz tones are smaller than during initial trials. A transient sensitization effect is seen in response to the 7.0 kHz tone. During discrimination trials, conditioned pupillary responses to the 2.0 kHz tone, designated as the CS+, are evident within 10 trials (block 5) and attain criterion by the 21st trial. In contrast, responses to the CS- initially decrease and never reach criterion. When the 7.0 kHz tone was then paired with brain stimulation, and the 2.0 kHz tone unpaired, CR's to the 2.0 kHz tone extinguish. Conversely, responses to the 7.0 kHz tone increased by the third trial block and reached criterion by the 25th trial (block 16). Clearly, the conditioned responses are specific to the CS.

4. Conclusions

The data summarized above demonstrate that we can obtain rapid learning in a restrained, awake cat, and that the US is rewarding. As outlined in the introduction, our next goal with this preparation is to record single neurons in the primary auditory cortex (AI) during acquisition of a conditioned response and to determine the effects of cholinergic agents on physiological plasticity which develops therein.

V. CONCLUSIONS

During the first year of this project, we have directed our efforts to three mutually supporting subjects which will be synthesized into studies of the involvement of cholinergic mechanisms, including anticholinesterases, in learning-induced plasticity of information processing in the auditory cortex.

Neurophysiological studies have determined that at least one third to one half of neurons within auditory cortex are sensitive to cholinergic agents applied by micropressure technology. The effects of cholinergic agonists can be blocked by antagonists. The cholinergic effect appears to be muscarinic. Importantly, the cholinergic system may have different effects on background activity and on different features of the response pattern to acoustic stimuli within the same neuron during the same period of investigation. This "mosaic" action of cholinergic agents underscores the essential need for thorough and quantitative analyses of cellular discharges, as opposed to characterizations limited to increases or decreases on the rate of discharge. The use of anticholinesterase agents during the coming year will provide important insights into the specificity of cholinergic mechanisms in neuronal discharge processes.

The development of waveform sorting of unitary discharges will, for the first time 1) permit simultaneous, rapid acquisition of relevant single cell data and 2) provide for future studies of the involvement of cholinergic mechanisms in neuronal functional networks (not part of the present project). We have developed a computer on-line real time waveform sorting algorithm which offers the

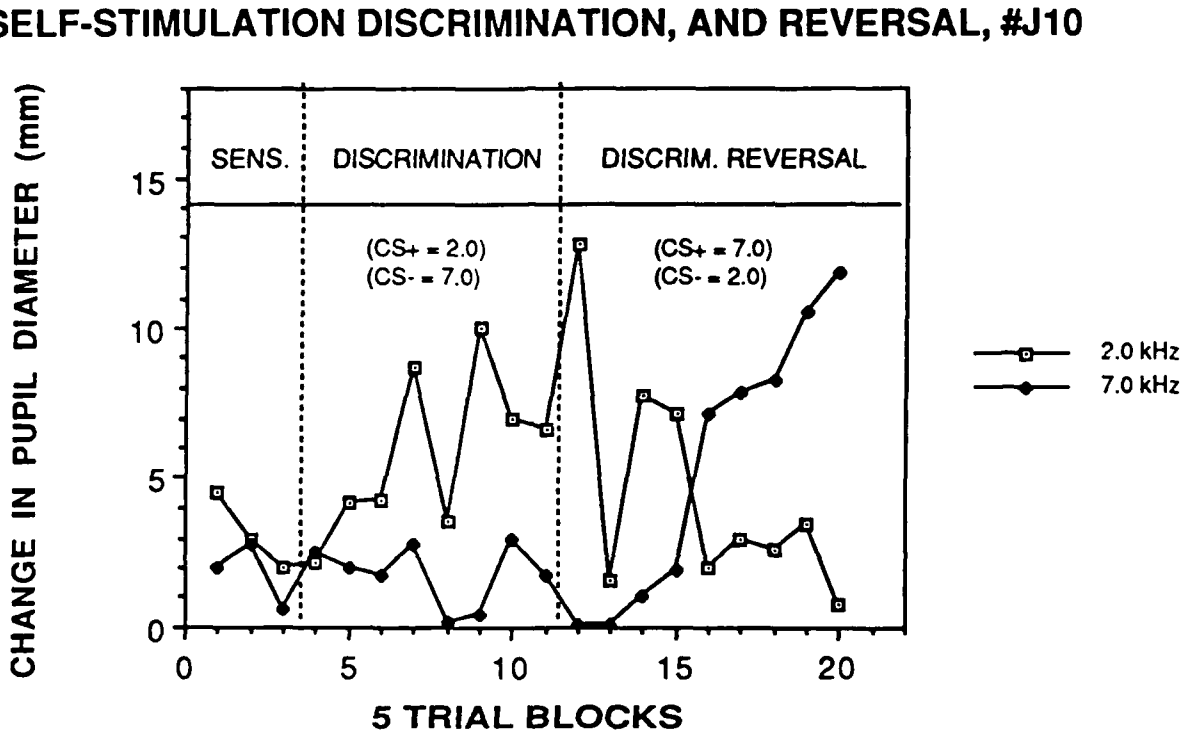

Figure 17. Discrimination and Discrimination Reversal with Hypothalamic Stimulation.

Hypothalamic stimulation, previously shown to be reinforcing by self-stimulation tests, can be used as the US in classical discriminative conditioning. Data presented here are for animal J10 during testing in which two tones were presented, only one of which was followed by the US. Note the increased pupillary conditioned responses to the reinforced stimulus (CS+) compared to the unreinforced tone (CS-), including discrimination reversal. These data illustrate that the relationship between the tone frequency and the positive brain stimulation are the determinants of the classical conditioned pupillary dilation response.

advantages of speed, ease of use, and the specification of statistical confidence limits. This is in the final stages of testing before deployment in actual experiments.

We have developed a behavioral learning model in which discriminated pupillary conditioned responses are acquired extremely rapidly using positive rewarding stimulation of the hypothalamus in a classical conditioning situation. This animal model will be used in combined cortical pharmacological learning studies during the second year of this project. In conjunction with waveform sorting, we should be able to obtain a significant amount of relevant information on the involvement of cholinergic mechanisms in cortical plasticity and information processing. This will provide critical data relevant to determining the mechanisms of action of existing and potential chemical warfare agents.

VI. RECOMMENDATIONS

At this time in the overall project, extensive recommendations cannot be made. This is particularly the case with reference to the problem of anticholinesterases because these agents are not scheduled for testing until Year 2. However, two recommendations can be advanced even at this relatively early stage of inquiry.

1. The investigation of sensory system physiology appears to be particularly advantageous. This is because the effects of agents upon the normal coding response properties of single neurons can be assessed rather directly. Use of normally transduced stimuli has interpretative advantages over the use of electrical stimulation because the latter cannot ordinarily be directly related to the normal processing of information. Furthermore, sensory system stimuli permit the investigation of all of the physical parameters of stimuli, thus permitting assessment of the effects of cholinergic agents on various dimensions of stimulus coding. In the present case, we have reported clear-cut effects of cholinergic agonists and blockers on specific components of the response of cortical cells to pure tone stimuli. It is recommended that consideration be given to further pursuing sensory processes.

2. We have found that the methods of data analysis are absolutely critical to understanding the effects of agents on neuronal activity. In particular, it appears essential to perform quantitative analyses on both background and evoked activity and, within the latter, to analyze all of the patterned components of responses to stimuli. It is recommended that consideration be given to 1) encourage comprehensive analyses of neuronal data, and 2) promote the development of analytical technologies which will facilitate such analyses, including computer-based data analysis systems. It is perhaps incumbent upon the investigator to demonstrate that simple data analyses are adequate to the problem at hand. The availability of information, guidance, and possibly appropriate computer programs should promote the USAMRDC mission as it can be approached by the field of neurobiology.

Future directions of the present line of investigation could determine the effects of systemic administration of cholinesterase blockers, as this would be the normal route of exposure in the field. The findings would provide the basis for understanding the effects of systemic administration of anticholinesterases, including agents such as sarin, soman, tabun, and VX. Furthermore, the

data from this project will also provide the basis for future studies of additional potential prophylactic agents, such as physostigmine and oximes.

It is clear that immediate solutions to critical problems in chemical defense are needed. It is equally clear that understanding the neurobiology of relevant agents constitutes a major challenge because of the inordinate complexity of the nervous system in general, the brain and the cerebral cortex in particular. Additionally, a review of the relevant literature reveals an alarming lack of foundational data, particularly for basic information processing and cholinergic mechanisms in awake animals, whose brains are functioning in a normal manner. In light of this critical mismatch between urgent need and lack of relevant understanding of the chemical systems that require management and control, it is essential that the best and most efficient use be made of all possibly relevant data, which is unclassified.

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